EFFECTS OF EXPOSURE TO 17α-ETHYNYLESTRADIOL DURING LARVAL DEVELOPMENT ON THE AFRICAN CLAWED FROG (*XENOPUS LAEVIS*) AND THE WOOD FROG (*RANA SYLVATICA*)

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy
In the Toxicology Graduate Program
University of Saskatchewan
Saskatoon
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ABSTRACT

The exposure of aquatic vertebrates to estrogenic compounds in the environment has become a concern in recent years. These compounds are present in the environment due to their presence in agricultural runoff, wastewater, pharmaceuticals, personal care products, and industrial products. Previous studies have shown that potent estrogens have the potential to feminize or demasculinize fish and amphibians, but the responses of many species to these compounds have not been fully characterized. The overall objective of this thesis was to determine the effects of exposure to 17α-ethynylestradiol (EE2), the estrogen analog commonly utilized in oral contraceptives, during the larval period on the sexual development, growth, metamorphosis, and transcriptional profiles of the African clawed frog (*Xenopus laevis*) and the wood frog (*Rana sylvatica*). To do so, *X. laevis* were exposed to 0.09, 0.84, or 8.81 µg EE2/L throughout the larval period and early post-metamorphic development (89 d) and *R. sylvatica* were exposed to 1.08, 9.55, or 80.9 µg EE2/L throughout the larval period until the climax of metamorphosis was reached (55-100 d). In *X. laevis*, exposure to all concentrations of EE2 tested resulted in significant delays in time required to complete metamorphosis of 15-23 d compared to the control treatment. In addition, exposure to all concentrations of EE2 tested resulted in significantly greater proportions of *X. laevis* individuals with a male genotype that displayed female, abnormal male, and mixed sex phenotypes. These individuals accounted for 83-93% of all genetic males in the EE2 treatments. While the development of genetic male *X. laevis* was affected by exposure to EE2, complete reversal of phenotypic sex was rare at the concentrations of EE2 tested, so it was not possible to calculate an EC_{50} for complete feminization. The EC_{50} for partial feminization was determined to be approximately 8.81 µg EE2/L. At the molecular level, exposure of *X. laevis* to EE2 resulted in alterations in abundances of transcripts of genes involved in endocrine processes, including steroid signaling and metabolism, vitellogenesis, testicular development, and thyroid hormone signaling, both during the larval period and after the completion of metamorphosis. In general, compared to the control treatment, transcripts of genes involved in testicular development and thyroid hormone signaling were suppressed during the larval period, and transcripts of genes involved in vitellogenesis were induced after the completion of metamorphosis. In addition, both genetic male and genetic female *X. laevis* exposed to EE2 displayed greater production of vitellogenin protein relative to

control individuals after the completion of metamorphosis. In *R. sylvatica*, exposure to all concentrations of EE2 tested resulted in significantly lesser proportions of phenotypic males which corresponded to greater proportions of female and mixed sex individuals. Phenotypic males accounted for 41%, 24%, 2.5%, and 0% of all individuals in the solvent control, 1.08, 9.55, and 80.9 µg EE2/L treatments, respectively. The EC_{50} for complete feminization for *R. sylvatica* was determined to be 7.7 µg EE2/L, and the EC_{50} for partial feminization was determined to be 2.3 µg EE2/L. At the molecular level, chronic exposure of *R. sylvatica* to EE2 resulted in induction of transcription of genes involved in vitellogenesis and the synthesis of cholesterol at the time of metamorphic climax. Although there were species-specific differences in effects between *X. laevis* and *R. sylvatica* after exposure to EE2, the overall response of both species was consistent with feminization or demasculinization, and both species were shown to be affected at concentrations of EE2 within the realm of environmental relevance.

ACKNOWLEDGEMENTS

I am grateful to have had the opportunity to complete my graduate studies under the supervision of Dr. John Giesy. In the past 10 years, he has served as a teacher and mentor throughout both my undergraduate and graduate careers, and I would not be defending my PhD had I not had his support. I am also grateful to Dr. Markus Hecker for serving as my unofficial co-supervisor. He has helped me in every facet of my graduate career, and I am a better scientist because of him. I would also like to thank my advisory committee members Dr. Barry Blakley, Dr. Mark Wickstrom, and Dr. Trent Bollinger for their comments and advice during my time at the University of Saskatchewan. I was lucky to have Dr. Natàlia G. Reyero Vinas serve as the external examiner for my defense, and I thank her for her flexibility and insightful comments.

I would like to acknowledge the financial support of the Toxicology Graduate Program, the College of Graduate Studies and Research, the Northern Ecosystems Toxicology Initiative, AREVA Resources, Natural Sciences and Engineering Research Council of Canada, the Canada Research Chair Program, and Western Economic Diversification Canada. There are many additional people who made my graduate studies possible. I would like to acknowledge those people that contributed to the completion of my research including Steve Wiseman, Eric Higley, Hong Chang, and Sara Pryce. I would also like to thank Jon Doering, Darren Nesbitt, Tim Tse, Shawn Beitel, and Brett Tendler for their technical assistance or advice. There isn't enough space to personally acknowledge every person at the Toxicology Centre who has impacted my life, and I feel blessed to have spent the last 5 years working with all of you. This is a fantastic place to do research with a fantastic group of people, and I will never forget the time I have spent at the University of Saskatchewan.

Lastly, I would like to thank my family (Mom, Dad, Autumn, Roger, Bailee, and Ally) for their love and for only asking me every once in awhile when I am going to be done with school. Most importantly, I want to thank Eric for being my partner in all things. The past 10 years have been great, but there are even better things to come for us, beginning with the birth of our beautiful daughter Natalie.

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LIST OF ABBREVIATIONS

µg/L = micrograms per liter

AMA = Amphibian Metamorphosis Assay

ASTM = ASTM International, formerly American Society for Testing and Materials

C = Celsius

cDNA = complimentary DNA

d = day

DAB = 3,3-diaminobenzidine

DO = dissolved oxygen

DNA = deoxyribonucleic acid

E2 = 17β-estradiol

EACs = endocrine active chemicals

EC_{50} = half maximal effective concentration

EE2 = 17α-ethynylestradiol

ER = estrogen receptor

FASTA = text-based format for representing nucleotide sequences

FETAX = Frog Embryo Teratogenesis Assay *Xenopus*

GO = gene ontology

HPLC-MS/MS = high performance liquid chromatography tandem mass spectrometry

hr = hour

IHC = immunohistochemistry/immunohistochemical

IU = International Unit

KEGG = Kyoto Encyclopedia of Genes and Genomes

LC_{50} = median lethal dose

mg/L = milligrams per liter

mS/cm = millisiemens per centimeter

min = minutes

n = number of samples

NF stage = Nieuwkoop-Faber stage

PCR = polymerase chain reaction

PGCs = primordial germ cells

qPCR = quantitative polymerase chain reaction

RNA = ribonucleic acid

RPKM = reads per kilobase of exon model per million mapped reads

T = testosterone

$t_{1/2}$ = half-life

TBS = Tris-buffered saline

USEPA = United States Environmental Protection Agency

V = Volts

VTG = vitellogenin

CHAPTER 1

General introduction

1.1. Abstract

Populations of amphibians from locations around the world are in decline. A number of causal factors, including habitat stressors, physical stressors, abiotic stressors, and chemical stressors, have been suggested as contributors to these decreasing populations. Among chemical stressors, estrogenic compounds have the potential to affect exposed populations of amphibians, especially since the sensitivity of some species of amphibian to estrogenic exposures during sexual differentiation and development has been well-documented. Thus, to expand scientific knowledge about the response of amphibians to estrogenic compounds, the responses of two species of amphibian, the African clawed frog (*Xenopus laevis*) and the wood frog (*Rana sylvatica*), to 17α-ethynylestradiol (EE2), the synthetic estrogen utilized in oral contraceptives, were characterized. The specific (null) hypotheses tested in this research were that 1) there was no effect of EE2 on gonadal development in *X. laevis* or *R. sylvatica*; 2) there was no effect of EE2 on mortality, growth, or time required to complete metamorphosis in *X. laevis* or *R. sylvatica*; 3) there was no effect of EE2 on the transcriptome of genetic male *X. laevis*; and 4) there was no effect of chronic exposure to EE2 on abundances of transcripts of genes of interest in *X. laevis* or *R. sylvatica*.

1.2. Declines of amphibian populations

Amphibians have gained popularity as research organisms in recent decades because populations of some amphibians have decreased rapidly (Stuart et al. 2004, Wake 2012). These declines are unique because species affected have ranged from rare (Crump et al. 1992) to common (Carrier and Beebee 2003) and have happened in both disturbed (Rannap et al. 2007) and relatively pristine areas (Crump et al. 1992, Bank et al. 2006, McMenamin et al. 2008). These declines have been attributed to several causes, including ultraviolet radiation (Blaustein et al. 1994, Blaustein et al. 1996), chytrid fungus (Lips 1999, Skerratt et al. 2007, Ryan et al. 2008), parasites (Johnson et al. 1999, Johnson et al. 2003), habitat loss or modification (Nystrom et al. 2007, Eigenbrod et al. 2008), invasive species (Knapp et al. 2001, Vredenburg 2004, Knapp et al. 2007, Witte et al. 2008,), overharvest (Harding 2000), acidification (reviewed by Pierce

1985), environmental contaminants (Davidson 2004, Relyea 2005, Boone 2008), weather patterns like global warming and El Niño (Pounds and Crump 1994, Stewart 1995, Whitfield et al. 2007, Laurance 2008), and various combinations of these stressors (Davidson et al. 2001, Pounds et al. 2006, Boone et al. 2007, Macias et al. 2007, Lips et al. 2008, Rohr et al. 2008a, Rohr et al. 2008b). At this time, the consensus of scientists is that declines in individual populations of frogs are probably due to different causative factors, not a single consistent stressor or stressors (Collins and Storfer 2003). Even though some of the declines have been well studied, most are still poorly understood.

The scope of the decline of amphibian populations worldwide is extensive. In 2004, *Science* published a much-cited seminal global amphibian assessment that estimated that up to one-third of amphibian species were in danger of extinction, and that 43% of currently described species were experiencing population declines (Stuart et al. 2004). In terms of the number of species threatened, endangered, or already extinct, populations of amphibians are decreasing more rapidly than those of either birds or mammals (Stuart et al. 2004). With close to half of all known species in decline, it is unlikely that natural population fluctuations could explain the problem of amphibian declines. In fact, current extinction rates are around 200 times the estimated historical rate (McCallum et al. 2007, Roelants et al. 2007), and if the outcome of cases of threatened and endangered species worldwide is worst-case, the extinction rate could be up to 45,000 times greater than the historical rate (McCallum et al. 2007).

Most species of frog begin life as free-swimming, aquatic larvae (tadpoles) and subsequently metamorphose into aquatic (i.e. members of the Pipidae family) or terrestrial (i.e. members of the Ranidae family) adults (Duellman and Trueb 1986). Adult frogs have varying amounts of contact with the aquatic environment, but all species with aquatic larvae must return to water to breed. Thus, frogs can be vulnerable to alterations in both the aquatic and terrestrial environments, and they are completely dependent upon the presence of proper aquatic habitat for successful reproduction. In addition to dependence upon the presence of aquatic habitats for reproductive purposes, frogs are also sensitive to the presence, or absence, of moisture in their local environments. Almost all frogs, even those that are mostly terrestrial, have a moist, permeable integument. Although they do possess lungs, frogs also respire through the skin and the majority of amphibians live in moist or wet environments (Harding 2000).

It has been suggested that amphibians are suffering widespread population declines because they are uniquely sensitive to both long- and short-term alterations in both aquatic and terrestrial environments (Blaustein and Wake 1990, Crump et al. 1992). These environmental alterations can be separated into four categories of possible proximal causal factors of amphibian decline which are: habitat stressors, physical stressors, biotic stressors, and chemical stressors (Linder et al. 2003). Habitat stressors include loss or permanent alteration of habitat, which can doom entire assemblages of species to extirpation or extinction. Unfortunately, habitats required by amphibians are often permanently altered or destroyed when land is utilized for urbanization, resource extraction, or agricultural activities (Sodhi et al. 2008). Physical stressors, such as UV-B radiation and changing weather patterns, can also harm amphibian populations. In particular, changes in weather patterns, especially rain and drought cycles, can lead to complete recruitment failure since many species are dependent upon certain moisture regimes to reproduce. One of the most well known species to have undergone a decline, the golden toad (*Bufo periglenes*), is presumed to be extinct after El Niño-induced drought derailed breeding efforts of the highly localized species (Crump et al. 1992). Biotic stressors include parasites and infectious diseases, such as chytridiomycosis. Chytridiomycosis infection has resulted in mass mortality in sensitive species of amphibians on at least four continents (Lips 1999, Skerratt et al. 2007, Ryan et al. 2008). Chemical stressors include contaminants introduced into the environment by human activities, such as pesticides, herbicides, de-icers, pharmaceuticals, and personal care products. Many researchers have suggested that the permeability of amphibian skin makes these animals exquisitely sensitive to waterborne pollution. In fact, the percutaneous passage of common environmental contaminants is as much as 300-fold more rapid across amphibian than mammalian skin (Quaranta et al. 2009). In addition, laboratory and mesocosm studies have indicated that amphibians may be suffering adverse effects from some current use herbicides, especially glyphosate (Relyea 2005, Dinehart et al. 2010). All four categories of stressor have the potential to impact populations of amphibians, either individually or in concert with one another.

1.3. Exposure of amphibians to contaminants

Amphibian toxicology has become an area of greater research focus recently (Sparling et al. 2000). A wide variety of species are being studied, although members of the Ranidae family and members of the Pipidae family, including *Xenopus laevis* and *Silurana (Xenopus) tropicalis*, dominate the literature. While amphibian toxicology spans a wide variety of topics, a few issues of toxicological concern have been highly publicized both in scientific and more general circles. Agricultural activity and the presence of pesticides and herbicides in the aquatic environment have been at least partially blamed for an increased susceptibility to parasite-induced abnormalities and mortalities (Koprivnikar et al. 2006, Rohr et al. 2008a, Rohr et al. 2008b). In addition, while controversial, the work of Hayes et al. (2002, 2003, 2006) has raised concern that frogs might be sensitive to the effects of endocrine active chemicals (EACs). EACs have the potential to alter the endocrine system and can belong to a broad range of chemical classes, including pharmaceuticals, personal care products, herbicides, and fungicides.

Some species of frogs are sensitive to exposure to certain EACs, including steroid hormones, during sexual determination and differentiation (Witschi et al. 1958, Mikamo and Witschi 1964, Pettersson et al. 2007, Gyllenhammar et al. 2008, Hogan et al. 2008, Hu et al. 2008). Although determination of sex is genetic in amphibians, phenotypic sex is plastic and can be altered by exposure to steroid hormones during this sensitive period (reviewed by Hayes 1998). Since sexual differentiation normally takes place during the larval period, aquatic tadpoles may be vulnerable to EACs present in surface water sources, such as steroid hormones released by wastewater treatment plants (Ankley et al. 2007). Previous studies have demonstrated that ecologically relevant concentrations of some EACs, particularly 17α-ethynylestradiol (EE2), can have effects on some species of frog (Park and Kidd 2005, Pettersson and Berg 2007).

1.4. Responses of amphibians to estrogenic compounds

Previous studies have focused upon effects of exposure to exogenous estrogenic compounds on amphibians, in part because amphibians seem to be particularly sensitive to these

compounds. Exposure to exogenous estrogen during the period of sexual determination and differentiation leads to female-skewed sex ratios in various species, including *X. laevis* (Chang and Witschi 1955, Mikamo and Witschi 1964), *S. tropicalis* (Pettersson et al. 2006, Gyllenhammar et al. 2008), and Northern leopard frogs (*Rana pipiens*) (Hogan et al. 2008) as well as numerous other species (Witschi 1951, Witschi 1958, Park and Kidd 2005, Pettersson and Berg 2007). Lesser fertility and alterations of the reproductive organs have been linked to exposure to estrogen as well, including impaired spermatogenesis (Lee et al. 2005, Hu et al. 2008), failure of oviduct formation (Pettersson et al. 2006), and development of mixed sex phenotypes (Park and Kidd 2005, Hu et al. 2008).

Although exposure to estrogens after the period of sexual determination and differentiation cannot cause reversal of sex, the estrogen receptor (ER) gene becomes auto-inducible, which means it is able to be up-regulated by the presence of endogenous or exogenous estrogens, sometime after this period (Tata et al. 1993). Once the ER gains this ability, exposure to estrogens activates typical estrogen-responsive pathways that are commonly used in studies of estrogenic compounds in a variety of vertebrate species (Garcia-Reyero et al. 2009, Watanabe et al. 2009, Villeneuve et al. 2012). The induction of estrogen-responsive genes presumably leads to the *in vivo* effects observed in aquatic vertebrates exposed to estrogenic substances, including reproductive failure and greater production of the yolk-precursor protein vitellogenin (VTG) (reviewed by Sumpter and Johnson 2008) as well as induction of secondary sexual characteristics, such as coloration of integument, that are typical of female frogs in males (Hayes and Menendez 1999).

Exposure to potent estrogens has also been shown to significantly delay metamorphosis in a number of species (Richards and Nace 1978, Hogan et al. 2008). In some species, metamorphosis is timed to optimize survival of juvenile frogs by occurring before ephemeral water sources dry but after tadpoles have been allowed to reach an appropriate size to feed and avoid predators (Duellman and Trueb 1986). The mechanism by which estrogenic substances inhibit larval development is not clear, but it is probably ultimately related to an alteration in thyroid hormone homeostasis. Since thyroid hormones are responsible for orchestrating metamorphosis (Shi 2000), alterations in thyroid hormone homeostasis would likely impact metamorphic endpoints.

Alterations in sex ratios, sexual development, and metamorphic schedule are all biologically relevant endpoints that can be impacted by exposure to potent estrogens. Changes in these factors have the capacity to alter individual and even population-level fitness in a negative way. Reductions in fitness have implications for survival of species (Flight 2010). Since populations of some amphibian species are already declining, reductions in the fitness of amphibians could be particularly harmful. Importantly, some of the effects of estrogenic chemicals on amphibians have been observed at concentrations that are within the realm of environmental relevance (Park and Kidd 2005, Pettersson and Berg 2007).

1.5. Use of 17α-ethynylestradiol as a model estrogen

17α-ethynylestradiol (EE2) is a useful model estrogen. While other estrogens, such as 17β-estradiol (E2), have similar effects to EE2 on phenotypic sex ratios of amphibians (Chang and Witschi 1955, Chang and Witschi 1956, Mikamo and Witschi 1964, Miyata et al. 1999), EE2 is preferred as a model estrogenic compound for a number of reasons. Specifically, EE2 is more convenient for waterborne exposures due to its greater relative persistence, which minimizes chemical concentration fluctuations (Mackenzie et al. 2003). In addition, humans use EE2 as a hormonal contraceptive and excrete both the parent compound and its metabolites into wastewater. These compounds are not fully removed by treatment facilities and are discharged to the aquatic environment in effluents (Ankley et al. 2007). Thus, EE2 is an environmentally relevant contaminant. In addition, EE2 has documented effects on some species of frog at environmentally relevant concentrations (Park and Kidd 2005, Pettersson and Berg 2007).

1.6. *Xenopus laevis* as a model amphibian species

X. laevis, the African clawed frog, is a commonly used model species in studies of early embryonic development, cell biology, and biochemistry. This species has been used to gather knowledge about the mechanisms responsible for orchestrating processes such as organogenesis, chromosome replication, and intracellular signaling (Burlibasa and Gavrila 2011). *X. laevis* is a

useful model because it is fully aquatic, easy to culture in the laboratory, can be induced to spawn at any time of the year, produces thousands of eggs per spawning event, produces eggs that are easy to manipulate, has fast external development, and has available transgenic technologies (Zhanfen and Xiaobai 2006).

X. laevis is a popular model species in toxicology for the same reasons that it is a popular species for the study of development. The embryos of *X. laevis* are often used to assess developmental toxicity/teratogenicity of chemicals of concern via use of the 96 h FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) assay (ASTM 2004). More recently, the USEPA has developed a 21 d metamorphosis assay utilizing *X. laevis* called the AMA (Amphibian Metamorphosis Assay) as part of its Endocrine Disruptor Screening Program (USEPA 2011). Although there is no standard procedure for toxicity testing that encompasses the entire larval period of *X. laevis*, there are instances of prolonged-FETAX exposures where the initial FETAX assay is followed by a grow-out period without chemical exposure that ends at metamorphosis (Gutleb et al. 1999, Turley et al. 2003, Gutleb et al. 2007). In addition, exposures that span the entire larval and metamorphic period have been performed with a variety of compounds, including herbicides and steroid hormones (Coady et al. 2005, Hu et al. 2008, Wolf et al. 2010).

The response of *X. laevis* to exposure to E2 during sexual differentiation is well characterized. Exposure to E2 during the sensitive period of sexual differentiation results in reversal of the phenotypic sex of genetic male animals to phenotypic females (Chang and Witschi 1955, Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Hu et al. 2008, Wolf et al. 2010). However, previous studies of sex reversal in *X. laevis* were limited by the inability to determine genetic sex in affected individuals. Sex reversal was either determined only after lengthy grow-out and back-breeding studies (Chang and Witschi 1955) or presumed based upon altered sex ratios (Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Wolf et al. 2010). Recently, a sex-linked gene was discovered in *X. laevis*, which makes it possible to determine an individual's genetic sex at any point during development (Yoshimoto et al. 2008). Thus, identification of sex-reversed individuals by use of a genotyping assay is now possible at any point in development after sexual differentiation has taken place.

While responses of *X. laevis* to E2 have been well characterized, there is little known about the effects of exposure to EE2 during sexual differentiation on this species. As mentioned previously, EE2 is more convenient than E2 for waterborne exposures due to its relatively

greater persistence (Mackenzie et al. 2003). In addition, it has been found to have effects on sexual differentiation and development of other closely-related species of amphibian, such as *S. tropicalis*, at environmentally relevant concentrations (Pettersson and Berg 2007).

1.7. *Rana sylvatica* as a non-model amphibian species

The wood frog (*Rana sylvatica*) is native to extensive portions of North America. It is common throughout much of its range (Harding 2000). The range of *R. sylvatica* is so large, in part, because it is able to survive even in very cold climates due to its tolerance of freezing (Layne and Lee 1995, Voituron et al. 2002). *R. sylvatica* ranges further north than any other reptile or amphibian and is the only frog found north of the Arctic Circle in North America (Conant and Collins 1998). In Saskatchewan, especially in the prairies surrounding Saskatoon, *R. sylvatica* is the only member of the Ranidae family that is commonly found.

R. sylvatica are explosive breeders that spawn only during a two week period in late winter or early spring. Spawning takes place mostly in ephemeral, fish-free water bodies. *R. sylvatica* egg masses are deposited in communal areas near the surface of water. Masses of eggs are tolerant of freezing episodes. Eggs can take up to a month to hatch depending upon temperature. *R. sylvatica* tadpoles are aquatic and complete metamorphosis in 6-15 weeks after hatching. After metamorphosis, juvenile *R. sylvatica* disperse into upland terrestrial habitats and return to the water only to spawn after they reach sexual maturity at 2-3 years of age. *R. sylvatica* overwinter in leaf litter or under logs and are tolerant to freezing due to their ability to cryo-protect themselves (Layne and Lee 1995).

Although *R. sylvatica* has been used in previous toxicological studies (Mackenzie et al. 2003, Relyea 2005, Hogan et al. 2006, Gahl et al. 2011), it is not typically used as a model species. Studies utilizing North American members of the Ranidae family are more commonly performed on the northern or southern leopard frog (*Rana pipiens* or *Rana sphenocephala*) (Tsai et al. 2005, Hogan et al. 2008, Storrs and Semlitsch 2008, Langlois et al. 2010), the bull frog (*Rana catesbeiana*) (Veldhoen and Helbing 2001, Gunderson et al. 2011), or the green frog (*Rana clamitans*) (Park and Kidd 2005, McDaniel et al. 2008). However, like many species of amphibian, studies that at least partially characterize the response of *R. sylvatica* to steroid

hormone exposure were performed decades ago (Witschi 1951). Exposure of *R. sylvatica* tadpoles to estrogenic compounds, including EE2, induced reversal of the phenotypic sex of genetic male animals (Witschi 1951). Some more recent research has focused on the response of larval *R. sylvatica* to exposure to EE2 during the period of sexual differentiation (Mackenzie et al. 2003), but this study suffered from design flaws, including a lack of replication. Therefore, there is a need to fully characterize the response of *R. sylvatica* to exposure to EE2 during the larval period.

1.8. Use of molecular techniques in toxicological studies

Most previous studies of effects of exposure of amphibians to estrogens on development have focused on traditional toxicological endpoints, such as mortality (Hogan et al. 2006), growth (Mackenzie et al. 2003, Hogan et al. 2006), and histological evaluation of phenotype (Miyata et al. 1999, Hogan et al. 2008, Wolf et al. 2010). There has been some effort to characterize the molecular basis of responses of amphibians to exposure to estrogens both during (Miyata et al. 1999) and after (Palmer et al. 1998, Rankouhi et al. 2005, Ratanasaeng et al. 2008) the period of sexual determination and differentiation, but these studies have been limited in scope, usually focusing on the abundance of transcripts of one gene, such as aromatase (*CYP19A*) (Miyata et al. 1999), or on concentrations in serum of one protein, such as VTG (Rankouhi et al. 2005). While information about responses of individual genes and proteins to estrogen exposure in amphibians is useful, it does not allow for further characterization of the pathways and processes that are impacted in these organisms after exposure to estrogens.

Advanced molecular technologies can make characterization of response pathways possible, especially at the level of transcription (Garcia-Reyero and Perkins 2011). For species for which information on the sequence of DNA is available, quantitative polymerase chain reaction (qPCR) arrays and microarrays can be used to determine relative abundances of transcripts for dozens to thousands of genes. Typically, qPCR is a more targeted approach used to characterize abundances of transcripts of fewer than 25 genes of interest, whereas microarrays have hundreds to thousands of genes that are pre-determined and usually less targeted. Both qPCR and microarrays require some knowledge of gene sequences beforehand, but next

generation deep sequencing technologies, like 454 sequencing and *Illumina* sequencing, can be used even for species for which limited information on sequences is available (Ekblom and Galindo 2008). These technologies can be used to sequence DNA of any type, including entire genomes and complimentary DNA (cDNA) libraries of transcriptomes (Droege and Hill 2008, Rothberg and Leamon 2008). While the quantitative capabilities of next generation sequencing technologies are not as established as those of qPCR or microarrays, the techniques are useful for expression analyses of entire transcriptomes, including in situations where sequence information is not available prior to the sequencing experiment (Chen et al. 2011).

Analyses and profiling of the abundances of gene transcripts, whether via microarray, qPCR array, or next generation sequencing, can be a useful tool for determining the responses of organisms to chemical exposure. Ideally, these data can be utilized to determine the molecular responses that then lead to adverse effects at the tissue, individual, or population level (Kramer et al. 2011, Nichols et al. 2011, Perkins et al. 2011). In the case of amphibians exposed to estrogens, molecular tools could be useful in characterizing the molecular changes that drive the adverse effects associated with exposure to estrogens, including reversal of phenotypic sex, abnormal sexual development, reproductive failure, and delays to metamorphosis.

1.9. Research objectives

The research detailed in the chapters of this thesis endeavors to characterize effects of exposure to EE2, a model potent estrogen, on sexual differentiation, sexual development, growth, metamorphosis, and molecular responses of two species of amphibian. Specifically, exposures of the African clawed frog (*X. laevis*) and the wood frog (*R. sylvatica*) to EE2 were performed at concentrations that elicited effects on phenotype. Other biological parameters, such as growth and time to metamorphosis, were observed to determine the potential effects of EE2 on these endpoints. After exposure, molecular techniques, including *Illumina* sequencing and qPCR, were used to determine molecular effects that were associated with exposure to EE2, both during and after the period of sexual determination and differentiation. Research objectives by chapter are provided in Table 1.1.

The specific research objectives evaluated and hypotheses tested were as follows:

Objective 1: Determination of the effects of exposure to EE2 during sexual determination and differentiation on gonadal development in the African clawed frog (*X. laevis*) and the wood frog (*R. sylvatica*).

Ho: *There is no effect of exposure to EE2 on gonadal development in* X. laevis *or* R. sylvatica.

Objective 2: Determination of the effects of exposure to EE2 during the larval period on mortality, growth, and time required to complete metamorphosis in *X. laevis* and *R. sylvatica*.

Ho: *There is no effect of exposure to EE2 on mortality, growth, or time required to complete metamorphosis in* X. laevis *or* R. sylvatica.

Objective 3: Determination of the transcriptional changes induced by exposure to EE2 during sexual determination and differentiation in genetic male *X. laevis*.

Ho: *There is no effect of exposure to EE2 on the transcriptome of genetic male* X. laevis.

Objective 4: Determination of alterations in abundances of transcripts of genes of interest caused by chronic exposure to EE2 during the larval period in *X. laevis* and *R. sylvatica*.

Ho: *There is no effect of chronic exposure to EE2 on abundances of transcripts of genes of interest in* X. laevis *or* R. sylvatica.

Table 1.1: Research objectives by chapter

CHAPTER	OBJECTIVES	CHAPTER DESCRIPTION
1	Introduction	Background information including declines of amphibian populations, amphibian toxicology, use of *Xenopus laevis* and *Rana sylvatica* in toxicological studies, EE2 as a model chemical, use of molecular tools in toxicological studies
2	Effects of 17α-ethynylestradiol on sexual differentiation and development of the African clawed frog (*Xenopus laevis*). *Published in Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology (2012, 156: 202-210)*	• Characterization of the effects of exposure to EE2 on gonadal development in *X. laevis* • Characterization of the effects of exposure to EE2 on time required to complete metamorphosis in *X. laevis* • Identification of vitellogenin protein present in the kidney-gonad complex of *X. laevis* exposed to EE2
3	Characterization of the transcriptional responses of genetic male *Xenopus laevis* tadpoles exposed to 17α-ethynylestradiol during sexual determination and differentiation	• *Illumina* sequencing and RNA-Seq analysis of the transcriptomes of genetic male *X. laevis* at NF stage 53 after exposure to EE2 • Determination of molecular processes affected by exposure to EE2 • Linkage of affected molecular processes with biological effects of exposure to EE2
4	Characterization of molecular changes in the liver of African clawed frogs (*Xenopus laevis*) exposed to 17α-ethynylestradiol throughout larval and early post-metamorphic development	• Determination of genes of interest for qPCR and primer design • Analysis of abundances of transcripts of genes of interest • Discussion of transcripts with altered transcripts involved in steroid signaling and metabolism, synthesis of cholesterol, and vitellogenesis
5	Effects of exposure to 17α-ethynylestradiol during development on growth, sexual differentiation, and hepatic gene expression in larval wood frogs (*Rana sylvatica*)	• Characterization of the effects of exposure to EE2 on gonadal development in *R. sylvatica* • Characterization of the effects of exposure to EE2 on mortality, growth, and time to metamorphic climax in *R. sylvatica* • Primer design for genes of interest for qPCR using *Illumina* sequencing data • Discussion of transcripts with altered abundances
6	General discussion	Summary of results reported in each chapter and key findings, comparisons between *X. laevis* and *R. sylvatica*, conclusions

CHAPTER 2[a]

Effects of 17*α*-ethynylestradiol on sexual differentiation and development of the African Clawed Frog (*Xenopus laevis*)

[a] This chapter has been published in Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology (2012, 156: 202-210), under joint authorship with Steve Wiseman (University of Saskatchewan), Eric Higley (University of Saskatchewan), Sara Pryce (University of Saskatchewan), Hong Chang (University of Saskatchewan), John P. Giesy (University of Saskatchewan), and Markus Hecker (University of Saskatchewan)

2.1. Abstract

Several studies have shown that exposure of amphibians, including the African clawed frog (*Xenopus laevis*), to potent estrogens at critical times during development results in feminization and/or demasculinization. However, genotyping of *X. laevis* has only recently become possible, so studies performed in the past were rarely able to make explicit linkages between genetic and phenotypic sex. Therefore, to further characterize this relationship, *X. laevis* tadpoles were exposed during development to 0.09, 0.84, or 8.81 μg/L 17α-ethynylestradiol (EE2) which is the estrogen analog commonly used in oral contraceptives. Exposure to all concentrations of EE2 tested resulted in significant delays in time to metamorphosis. Genotyping showed that genetic sex ratios were similar among treatments. However, morphological evaluation revealed that a significant number of individuals with a male genotype displayed intersex and abnormal phenotypes. Additionally, both genetic males and females exposed to EE2 exhibited greater production of vitellogenin relative to the respective controls. Since estrogens function downstream of the initial molecular signals of sexual differentiation, it is likely that genetic male animals received mixed endogenous male and exogenous female signals that caused disordered sexual development. The production of vitellogenin was probably temporally separated and independent from primary effects on sexual differentiation, and may have contributed to the delays to metamorphosis observed in individuals exposed to EE2.

2.2. Introduction

The presence of estrogenic chemicals in the environment has become a significant cause for concern in recent years. Estrogenic substances are known to enter the aquatic environment through sources such as the discharge of liquid effluents from wastewater treatment facilities (Ankley et al. 2007). Exposure to these chemicals has been shown to result in feminization and/or demasculinization of aquatic vertebrates, mostly fish (Jobling et al. 1998, Kidd et al. 2007, Sumpter and Johnson 2008). Laboratory studies have shown that these effects are consistent in other aquatic vertebrates, including various amphibians (Chang and Witschi 1955, Witschi et al. 1958, Hogan et al. 2008). Estrogenic compounds, including endogenous

hormones, pharmaceuticals, and industrial products, share a similar mode of action, which is the ability to bind to and agonize the estrogen receptor, thereby inducing expression of estrogen responsive genes (Boelsterli 2003). Induction of these genes presumably causes *in vivo* effects observed in aquatic vertebrates exposed to estrogenic substances, including reproductive failure, formation of mixed sex or sex reversed gonads, and increased production of the egg yolk protein vitellogenin (reviewed by Sumpter and Johnson 2008). Some amphibians are in decline worldwide (Stuart et al. 2004), and it has been suggested that exposure to estrogenic substances could be contributing to amphibian declines in some cases. As such, further investigation of the effects of estrogens on reproductive endpoints in the amphibians is warranted.

Amphibians, including the African clawed frog (*Xenopus laevis*), are relatively sensitive to steroid hormones when exposed during the sensitive period of sexual determination and differentiation of gonads (Chang and Witschi 1955, Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Lutz et al. 2008). Exposure to sufficient doses of potent estrogens during this period causes disordered sexual development and reversal of the male phenotype of genetic males to a female phenotype (Chang and Witschi 1955). However, because it was not possible to assign genetic sex in *X. laevis* until the recent discovery of the gene *DM-W*, a sex-linked gene located on the W chromosome (Yoshimoto et al. 2008), conclusions about effects of estrogens on sexual development of *X. laevis* have historically been drawn only after lengthy grow-out and back-breeding experiments (Chang and Witschi 1955, Chang and Witschi 1956) or inferred based on altered sex ratios in treated groups (Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Lutz et al. 2008). This limitation has led to some uncertainty in the interpretation of results of experiments in which juvenile *X. laevis* were exposed to putative estrogenic compounds.

X. laevis has a gonochoristic ZW system that determines genetic sex. In *X. laevis*, females are the heterogametic sex (ZW karyotype) and males are the homogametic sex (ZZ karyotype). The Z and W chromosomes are morphologically indistinguishable (Schmid and Steinlein 1991), which makes it impossible to differentiate between male and female genotypes via karyotyping. However, the discovery of *DM-W*, a W-linked gene, has made it relatively easy to assign genetic sex by use of molecular techniques. As such, *DM-W* affords researchers the opportunity to perform sex reversal experiments with *X. laevis* with the ability to draw conclusions on the basis of genetic sex at any point during development.

The role of *DM-W* in development of the female phenotype is not completely clear. *DM-W* is expressed transiently during development of females (Yoshimoto et al. 2008) and it is likely a transcription factor with a DM-type DNA-binding domain (Yoshimoto et al. 2010). The *DM-W* transcription factor has a DNA-binding sequence that is similar to that of the known testis determining factor *DMRT1,* and it binds to the same genes as *DMRT1* (Yoshimoto et al. 2010). It is possible that *DM-W* antagonizes the action of *DMRT1*, thereby allowing development of a female phenotype. *In vivo* transfection of genetic male tadpoles with a *DM-W* expression vector seems to partially support this hypothesized scenario, since introduction of the vector can induce formation of some primary ovarian structures (Yoshimoto et al. 2008). However, transfection with *DM-W* is not capable of causing complete reversal of the phenotypic sex. This observation indicates that some other factors, either W-linked or specific to the ZZ chromosome complement, might also influence primary sexual differentiation (Yoshimoto et al. 2008).

Artificial expression of *DM-W* cannot fully feminize male *X. laevis*, but males can be completely feminized by exposure to potent estrogens during the period of Nieuwkoop-Faber (NF) stages 50-52 (Nieuwkoop and Faber 1994) (Villalpando and Merchant-Larios 1990, Miyata et al. 1999). If treatment is halted at NF stage 51, some mixed sex gonads result (Miyata et al. 1999), and the developing gonad is still sensitive to partial feminization by exposures initiated at or before NF stage 54 (Villalpando and Merchant-Larios 1990). Exposure to estrogens later than NF stage 54 does not affect the phenotype. To avoid missing the sensitive period during development, some studies have used exposure throughout larval development with no adverse effects on survival or growth of the tadpoles (Lutz et al. 2008).

The concentration of potent estrogen required to induce complete change of a genetic male to the female phenotype is less clear than the timing of exposure to estrogens that causes effects. Estrogens with different potencies, such as 17β-estradiol (E2) and estradiol benzoate, have different effective doses. In addition, even replicate studies with the same estrogen exhibit different effective concentrations for feminization (Lutz et al. 2008). The initial work performed by Chang and Witschi (1955, 1966) indicated a slightly male-biased sex ratio due to exposure to 10 µg E2/L, a male:female ratio of 3:22 after treatment with 25 µg E2/L, and complete reversal of phenotypic sex at 50 µg E2/L. Results of more recent experiments that utilized continuous flow-through renewal systems have demonstrated that the EC_{50} for feminization of genetic males

was as little as 0.12 µg E2/L (Lutz et al. 2008) with a mean value of approximately 0.2 µg E2/L (Wolf et al. 2010).

Due to their presence in effluents from wastewater treatment plants, both E2 and its synthetic analog 17α-ethynylestradiol (EE2) are considered environmentally relevant chemicals of concern (Ankley et al. 2007). The current study was designed to utilize the model estrogen EE2 to cause disordered sexual development in *X. laevis* and to definitively identify the genetic sex of impacted animals by presence or absence of the sex-linked gene *DM-W*. Much of the previous work performed with *X. laevis* has used E2 as a test chemical, so EE2 was used as a model chemical to expand comprehension of the response of *X. laevis* to different potent estrogens. EE2 is the synthetic estrogen used in human oral contraceptives and is as much as two times as potent as E2 *in vitro* (Folmar et al. 2002) and twice as persistent as E2 in the environment (Ying et al. 2002). Thus, it was predicted that if concentrations of EE2 equivalent to E2 concentrations that caused effect were used that biological effects would be similar or greater. Based on results previously published in the literature, the nominal concentrations of EE2 chosen for the current study, 0.1, 1, and 10 µg EE2/L, were expected to cause few, moderate, and severe effects (complete reversal of male phenotypes to female phenotypes), respectively. Concentrations of EE2 chosen for the current study were based upon their ability to elicit effects on phenotype, but the least dose was approximately 3-fold greater than the range of estrogen equivalents (about 5-30 ng/L) that would be expected in a natural watershed (reviewed by Kidd et al. 2007).

2.3. Materials and methods

2.3.1. *Xenopus laevis*

Prior to commencement of research, approval for the use of animals and all experimental procedures was obtained from the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan (Animal Use Protocol #20090066). Sexually-mature, adult *X. laevis* were purchased from Boreal Laboratories (St. Catharine's, ON, Canada) and acclimated to laboratory conditions (18 ± 2 °C water temperature; 12:12 light-dark

cycle; fed Nasco frog brittle [medium nuggets] (Salida, CA, USA) *ad libitum* daily) for one month. For breeding, both male and female *X. laevis* were primed with 500 IU of human chorionic gonadotropin (EMD Biosciences, San Diego, CA, USA) dissolved in Nanopure water (Thermo Scientific, Asheville, NC, USA) injected into the dorsal lymph sac about 24 hr before spawning was to occur. The following day, female *X. laevis* were injected with 1000 IU human chorionic gonadotropin, and males were injected with 500 IU human chorionic gonadotropin. Breeding pairs were then transferred to breeding tanks with 20 °C water and dark covers and left to spawn overnight. Eggs were collected from tanks about 8 hr post-oviposition. They were then dejellied in a 2% solution (pH=8.1) of L-Cysteine (Sigma, St. Louis, MO, USA), rinsed in FETAX solution (ASTM 2004; 625 mg/L NaCl, 96 mg/L $NaHCO_3$, 30 mg/L KCl, 15 mg/L $CaCl_2$, 60 mg/L $CaSO_4 \cdot 2H_2O$, and 75 mg/L $MgSO_4$ dissolved in reverse osmosis water and pH adjusted to 7.6-7.9; individual salts purchased from Sigma), and placed into Petri dishes containing FETAX solution. Dejellied eggs were then sorted, and unhealthy and unfertilized eggs were discarded.

2.3.2. 17α-ethynylestradiol exposure

Healthy fertilized eggs (50 per tank) were placed into 6L of FETAX with the appropriate nominal concentration of EE2 (0.1, 1, or 10 µg/L; Sigma) dissolved in an ethanol carrier (Commercial Alcohols 95% ethyl alcohol, Toronto, ON, Canada) at about 13 hrs post-oviposition. The final concentration of ethanol in treatment tanks, including solvent control tanks, was 0.0025%. Each treatment was run in triplicate. Subsamples of tadpoles were removed during the exposure period for molecular analysis that is not presented here. After removal of subsamples, 15 tadpoles remained in each tank and were allowed to complete metamorphosis.

Tadpoles were fed *ad libitum* daily with a slurry of Nasco frog brittle (tadpole powder). At completion of metamorphosis, froglets were fed Nasco frog brittle (small nuggets). Each day, a 50% static water renewal was performed on each tank. Basic water quality measurements (temperature, DO, pH, conductivity) were collected daily with an YSI Quatro Multi-Parameter probe (Yellow Springs, OH, USA). Concentrations of ammonia nitrogen, nitrate nitrogen, and

nitrite nitrogen were monitored weekly with Lamotte colorimetric kits (Chestertown, MD, USA). At the beginning of the experiment, the percent of eggs that hatched was determined. Mortality of tadpoles and frogs was recorded daily thereafter. As animals matured, time to completion of metamorphosis was recorded. Some of the animals in the EE2 treatments failed to complete metamorphosis during the experiment, which was recorded during experiment termination.

2.3.3. Analysis of 17α-ethynylestradiol concentrations in treatment water

Concentrations of EE2 in exposure water were monitored periodically during the experiment via high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) to validate that expected nominal values were approximated. EE2 was quantified as has been described elsewhere (Chang et al. 2010). Briefly, samples of whole water were spiked with a deuterated internal EE2 standard (C/D/N Isotopes, Inc., Pointe-Claire, QC, Canada), extracted two times with hexane and then concentrated under nitrogen. Dried organic extracts were derivatized with dansyl chloride, re-extracted with hexane, dried under nitrogen, and then reconstituted in acetonitrile. Analytical detection was conducted using an Agilent 1200 series HPLC system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole MS/MS system (PE Sciex, Concord, ON, Canada). Both the LC and the mass spectrometer were controlled by AB Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA, USA).

2.3.4. Termination of exposure and determination of phenotypic sex

X. laevis were exposed to EE2 for 89 d. At termination of the exposure, individuals that had completed metamorphosis were euthanized in an overdose of MS-222 (Sigma). Each individual was weighed, measured, and gross phenotypic morphology was determined with a dissecting microscope (Olympus SZ40, Center Valley, PA, USA). Selected organs were removed for subsequent molecular analysis that is not detailed here. Then, the entire animal, including the gonads inside the body cavity, was placed into 10% formalin and fixed for at least 24 hr.

After proper fixation, gonads were re-observed for phenotype and photographed with a digital camera (Carl Zeiss AxioCam ICc3, Toronto, ON, Canada) attached to the dissecting microscope. Then, the gonad-kidney complex was removed, placed into a tissue cassette, and processed in a MVP1 Modular Vacuum Processor (Instrumentation Laboratory, Bedford, MA, USA) by use of standard protocols. Tissues were embedded in paraffin blocks and serially sectioned at 7 µm intervals. Tissue sections were placed onto a 37 °C water bath and then picked up on Superfrost Plus slides (ThermoScientific, Pittsburgh, PA, USA). Slides were allowed to dry on a slide warmer overnight and then stored in slide boxes until staining.

For each individual, the entire gonad or at least three slides with gonad sections, including two slides from the apices of the gonad and one slide from the medial gonad, were stained with hematoxylin and eosin by use of standard protocols. After staining, sections were covered with cover slips and Microkitt xylene-based mounting medium (Serum International, Inc, Laval, QC, Canada) and allowed to dry for at least 24 hr.

Gonadal histology was evaluated using a Carl Zeiss Axio Observer.Z1 microscope equipped with a digital camera (Carl Zeiss AxioCam ICc1) and interfaced to a computer. Representative images were recorded and saved using Axiovision LE 4.7.2 software (Carl Zeiss). Slides were coded so that the observer was blinded to the treatment group each individual belonged to. Each slide was examined for the presence of male and female type tissues, and it was assessed whether those tissues were of normal morphology or not (Ankley et al. 2006). Individuals were classified as male, female, mixed sex, or abnormal male (Hecker et al. 2006). Abnormal males were characterized by a lack of spermatocysts and/or abnormal shape of the testis.

2.3.5. Vitellogenin immunohistochemistry

An unknown proteinaceous fluid was observed in and around the sections of kidney-gonad complexes evaluated for phenotype in many of the individuals from the EE2 exposure groups. This fluid was hypothesized to be vitellogenin (VTG) (Ankley et al. 2006), so an immunohistochemical (IHC) method was developed to determine the identity of this unknown protein. The method was based on standard IHC procedures and was optimized for use in *X.*

laevis. The optimized procedure for histological slides was as follows: 2x2 min xylene; 2x2 min 100% ethanol; 2 min 95% ethanol; 2 min 70% ethanol; 20 sec distilled water; 7 min 1:50 Proteinase K (DakoCytomation, Burlington, ON, Canada) in a humid chamber; 2x2min Tris-buffered saline (TBS); 10 min 3% H_20_2 (Sigma) in a humid chamber; 2x2min TBS; 30 min DakoCytomation Protein block, serum free in a humid chamber; 24 hr primary antibody (Biosense VTG-13, polyclonal rabbit anti-seabream vitellogenin, Bergen, Norway) diluted 1:250 with 0.01 mol/L Tris-HCl with 0.3% Triton-X (Sigma) in a humid chamber at 4˚C; 2x2 min TBS; 30 min horseradish peroxidase conjugated secondary antibody (DakoCytomation polyclonal anti-rabbit immunoglobulins/HRP) diluted 1:200 in Tris-HCl/Triton X buffer in a humid chamber; 2x2 min TBS; 10 min DAB working substrate (DakoCytomation); 2 min running tap water; one dip in each graduated ethanol (70, 95, 100, and 100%); 1 min 100% ethanol; 2x2 min xylene. After immunostaining, cover slips were applied with Microkitt mounting medium, and slides were allowed to dry for at least 24 hr. Slides were observed and representative images recorded as detailed above for hematoxylin and eosin stained slides.

2.3.6. Determination of genetic sex

In *X. laevis*, the *DM-W* gene can be used as a marker of female genetic sex, and its absence is indicative of male genetic sex. A simple PCR assay has been developed to determine genetic sex in *X. laevis* individuals (Yoshimoto et al. 2008). Briefly, the multiplex assay can amplify two differentially sized gene fragments, a 260 bp fragment of *DM-W* and a 208 bp fragment of *DMRT1*. The *DMRT1* gene serves as an internal control gene that is amplified in both males and females because this gene resides on an autosome. The *DM-W* gene is only amplified in genetic females that possess a W chromosome. The assay uses a genomic DNA sample and, thus, can be performed on any tissue, including pieces of tadpole tails and toe clips.

Genomic DNA was isolated from a tissue sample taken from the leg of each frog with a QIAamp DNA Mini Kit (Qiagen, Toronto, ON, Canada). DNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples of genomic DNA were used as a template in the genetic sexing assay described above. The primers were 5'-CCACACCCAGCTCATGTAAAG-3' and 5'-GGGCAGAGTCACATATACTG-3' for *DM-W*

and 5'-AACAGGAGCCCAATTCT-3' and 5'-AACTGCTTGACCTCTAATGC-3' for *DMRT1*. Reactions were conducted in PCR tubes and consisted of 0.5 µM forward and reverse *DMRT1* and *DM-W* primers (Invitrogen, Burlington, ON, Canada), 5 mM $MgCl_2$ (Biorad, Mississauga, ON, Canada), 0.5 mM dNTPs (Biorad), 1x Taq buffer (Biorad), 2.5 U Taq polymerase (Biorad), and 1 µM genomic DNA template and run on a Mycycler PCR machine (Biorad; 95 °C for 5 min; 40x of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min; 72 °C for 7 min). PCR products were visualized on gels consisting of 2% agarose (Agarose PCR Plus, EMD Chemicals Inc., Gibbstown, NJ, USA) in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) with 1 µg/mL ethidium bromide (Sigma). Gels were run at 50V for at least 2 hr to accomplish adequate separation of product bands. Images of gels were captured on a Versadoc MP4000 imager (Biorad) using UV light. Individuals whose genomic DNA amplified both *DMRT1* and *DM-W* were classified as genetic females while individuals that only amplified *DMRT1* were classified as genetic males (Figure 2.1).

2.3.7. Statistics

Statistical tests were performed using IBM SPSS 19 software (IBM, Armonk, NY). Treatment means are expressed as mean ± S.D. throughout. Statistical significance was defined as $p \leq 0.05$. Data for percent hatch and mortality were analyzed with Pearson Chi-Square tests to determine differences among treatments. Data for the proportion of tadpoles that completed metamorphosis during the experiment were analyzed with Fisher's Exact test. The mean number of days to metamorphosis was determined for each treatment using a survival analysis to allow inclusion of animals that failed to complete metamorphosis during the experiment. The data for mean number of days to metamorphosis were normally distributed (Shapiro-Wilk test) and had homogenous variances (Levene's test), so they were analyzed with an ANOVA with a post-hoc Tukey's test to determine differences among treatment means. Since exposure to EE2 significantly impacted time to metamorphosis and all animals were euthanized at the same time, morphometric (weight and length) data were not included in the current analysis due to shorter grow-out times for animals in the EE2 treatments. Data for genetic sex were analyzed with a Pearson Chi-Square test to determine differences among treatments and a standard Chi-Square

test to determine overall and treatment specific similarity to a 50:50 sex ratio. Data for phenotypic sex of genetic males were analyzed with Fisher's Exact test to determine whether the proportion of individuals in each phenotype category was similar among treatments. The p-value for the Fisher's Exact test was significant, so post-hoc analysis of the phenotypic sex data was completed by comparing the standardized residual for each phenotype category in each treatment to a critical value (α=0.05, critical value=$\pm$1.96; α=0.01, critical value=$\pm$2.58). If the residual exceeded the critical value, that phenotype category was assigned a significance value based upon the magnitude of the residual (i.e. $p<0.05$ where the standardized residual was greater than $\pm$1.96).

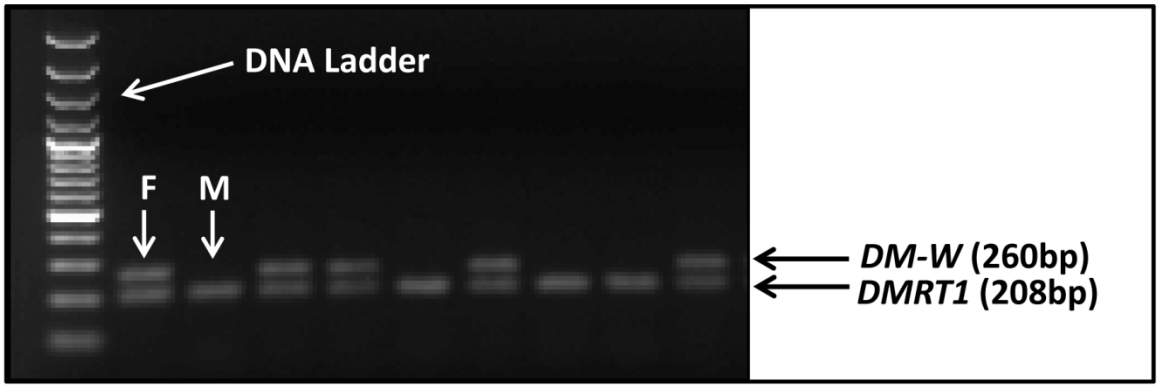

Figure 2.1: Gel depicting genotypes of *Xenopus laevis.* Representative image of an agarose gel depicting the genotype of 9 *X. laevis* individuals. The band at 260 bp is a fragment of the sex-linked gene, *DM-W*, and the band at 208 bp is a fragment of the autosomal gene, *DMRT1*. Individuals with two bands amplified are genetic females (F), while those with only one band are genetic males (M).

2.4. Results

2.4.1. Water quality and validation of 17α-ethynylestradiol concentrations

Water quality variables over the course of the experiment were all within an acceptable range for culturing amphibians. Average values were temperature of 22.5 ± 0.1 °C, conductivity of 1.69 ± 0.01 mS/cm, 7.1 ± 0.2 mg dissolved oxygen/L, pH of 7.7 ± 0.1 standard units, and 0.03 ± 0.02 mg ammonia/L. Nitrite was never detected above the method detection limit of 0.02 mg/L. Nitrate was detected in 17% of samples at concentrations of 0.25 mg nitrate/L, which was the least concentration of nitrate detectable by the kit used. The remaining 83% of samples did not have detectable nitrate concentrations (<0.25 mg nitrate/L).

Concentrations of EE2 in treatments and the absence of detectable EE2 in control groups were confirmed. The limit of quantification for quantification of EE2 by LC-MS/MS was 0.02 µg EE2/L. EE2 was not detected at any point during the experiment in control or solvent control tanks. Nominal concentrations of EE2 treatments were 0.1, 1, and 10 µg EE2/L, and actual concentrations were 0.09 ± 0.005, 0.84 ± 0.06, and 8.81 ± 1.25 µg EE2/L immediately following water changes, respectively. To determine whether concentrations of EE2 remained stable over the 24 hr period between water renewals, concentrations were also determined just prior to water change. After 24 hr, average concentrations of EE2 in water decreased to 0.07 ± 0.005, 0.48 ± 0.10, and 6.37 ± 1.25 µg EE2/L in the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively. These values correspond to half-lives ($t_{1/2}$) of 2.8, 1.2, and 2.1 d, respectively. Since EE2 concentrations just prior to water change were not monitored as often as concentrations immediately following water change, validated concentrations of EE2 after water change are used hereafter to designate treatment groups.

2.4.2. Percent hatch, mortality, and days to metamorphosis

On average, 92% of *X. laevis* eggs survived to hatching. Within treatment, the number of eggs that hatched ranged from 90% in control water to 93% in solvent control water, 0.84 and 8.81 µg EE2/L. There were no differences among treatments in percent hatch (Pearson Chi-

Square, p=0.888). Over the course of the experiment, mortality of tadpoles and frogs ranged from 4% in 0.84 µg EE2/L to 10% in 0.09 µg EE2/L. Overall average mortality was 8%. There were no differences among treatments in mortality (Pearson Chi-Square, p=0.315). Although there is no mortality guideline for the type of experimental design utilized in the current study, mortality was at or below the standard set for a valid study by the US Environmental Protection Agency (≤10%) for the 21-day Amphibian Metamorphosis Assay utilizing *X. laevis* (USEPA 2011).

Some tadpoles exposed to EE2, but not in the control or solvent control treatments, failed to complete metamorphosis during the course of the experiment. The percentage of tadpoles completing metamorphosis was 100%, 100%, 86%, 79%, and 52% in the control, solvent control, 0.09 µg EE2/L, 0.84 µg EE2/L, and 8.81 µg EE2/L treatments, respectively. There was a statistically significant difference in the proportion of tadpoles in each treatment that completed metamorphosis (Fisher's Exact Test, p<0.001).

The mean number of days required to complete metamorphosis ranged from 65 d in control animals to 88 d in animals grown in water containing 8.81 µg EE2/L. A survival analysis was used to evaluate time to complete metamorphosis in a manner that could include those individuals that failed to complete metamorphosis during the experiment. A survival analysis was completed for each replicate tank, and replicate values were averaged to determine mean time to complete metamorphosis in each treatment. There was a statistically significant (ANOVA, p<0.001), dose-dependent trend of greater mean times to complete metamorphosis with the addition of both solvent and increasing concentrations of EE2 (Figure 2.2). Individuals in all of the EE2 treatments had significantly delayed metamorphosis compared to control animals (p=0.008, p=0.003, and p<0.001 for the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively). Time to metamorphosis of individuals exposed to the solvent control was not significantly different from that of from control frogs (p=0.558) or frogs exposed to 0.09 µg EE2/L (p=0.083), but did differ from those exposed to 0.84 and 8.81 µg EE2/L (p=0.029 and 0.002, respectively).

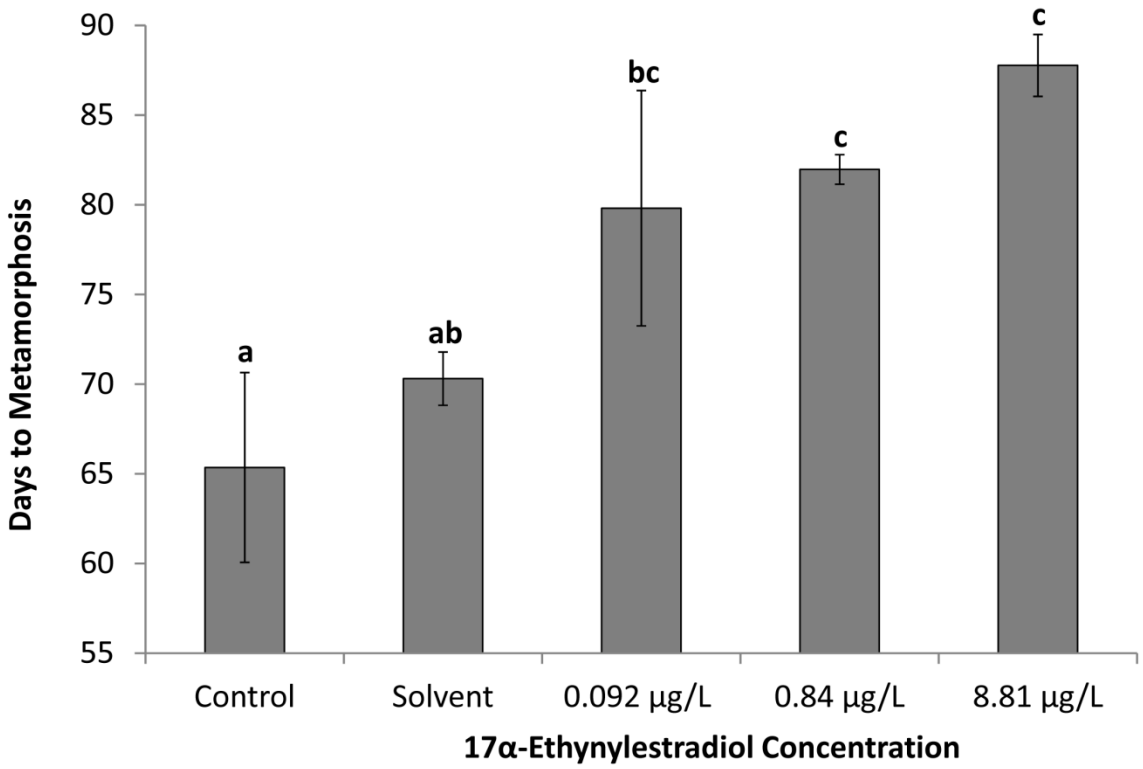

Figure 2.2: Mean number of days required to complete metamorphosis by *Xenopus laevis* tadpoles exposed to 17α-ethynylestradiol during larval development. Data are presented as number of days to metamorphosis ± S.D (*n*=3 replicate tanks for each treatment). Exposure to 17α-ethynylestradiol significantly delayed metamorphosis by up to 23 d (ANOVA, $p<0.001$). Significant differences among treatments are denoted by different letters.

2.4.3. Genetic sex ratios

There were no statistically significant differences in genetic sex ratios among treatments (Pearson Chi-Square, p=0.860; Figure 2.3). The overall male:female genetic sex ratio was 49:51, which did not differ from a 50:50 sex ratio (Chi Square, p=0.647). In addition, the genetic sex ratio in each treatment did not differ significantly from a 50:50 sex ratio (p=0.763, 0.876, 0.480, 0.493, and 0.513 for the control, solvent control, 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively). The sex ratio in the 0.09 µg EE2/L treatment appeared slightly female-biased compared to the other treatments, but the difference was not significant (Figure 2.3). Sex ratios in this treatment were also more variable than those in other treatments. Therefore, analysis of phenotypic sex was performed on genetic females and males separately to avoid biasing the results due to the slightly uneven genetic sex ratios.

2.4.4. Phenotypic sex ratios

All animals were assigned to one of 4 phenotypes based upon gross and histological analyses: female, male, abnormal male, or mixed sex (Figures 2.4 and 2.5). Initial gross morphological phenotyping indicated that as many as 80% of genetic male animals in the 8.81 µg EE2/L treatment developed as phenotypic females. However, subsequent histological analysis revealed that some individuals that had a gross morphology more consistent with a female than male phenotype were actually genetic males that had developed abnormally (Figure 2.5D), were mixed sex (Figure 2.4C), or were exhibiting abnormal protein production (Figure 2.7), which was characterized by an eosin-stained proteinaceous fluid present in and around the kidney-gonad complex of both genetic males and females in all EE2 treatments and is discussed below. In some cases, the number of phenotypic female animals decreased by up to 40% after histological analysis. For example, with only gross morphology to guide conclusions, initial phenotypic sex ratios in the 8.81 µg EE2/L treatment appeared to be 90% female, 5% male, and 5% unknown/ambiguous, inclusive of both genetic males and females. After histological analysis, it was found that only 52% of animals were phenotypic females, 19% were mixed sex, 24% were abnormal males, and 5% were males (Figure 2.6).

Although some of the genetic female frogs exposed to EE2 exhibited abnormal protein production, they were all classified as phenotypic females possessing histologically normal ovarian tissue. Genetic male animals from all treatments were classified as either normal males with normal testicular tissue, abnormal males with abnormal testicular tissue, mixed sex with both testicular and ovarian tissue, or phenotypic female (sex-reversed male) with normal ovarian tissue (Figures 2.4 and 2.5). While the exact phenotype of abnormal male animals varied among individuals, these individuals all had recognizable testicular tissue and no primary oocytes present. The testicular tissue present exhibited abnormalities ranging from diffuse shapes to internal cavities to abnormal cellular morphology (absence of spermatocysts).

Among genetic males, there were significant differences between treatments in the proportion of male, abnormal male, mixed sex, and female phenotypes (Fisher's Exact test, $p<0.001$) (Figure 2.6; Table 2.1). Normal males accounted for 100%, 95%, 17%, 7%, and 8% of total genetic males in the control, solvent control, 0.09 µg EE2/L, 0.84 µg EE2/L, and 8.81 µg EE2/L treatments, respectively. Statistically, normal males were over-represented in control and solvent control treatments and under-represented in all EE2 treatments ($p<0.05$ for all). Abnormal males accounted for 0%, 5%, 72%, 67%, and 42% of total genetic males in the control, solvent control, 0.09 µg EE2/L, 0.84 µg EE2/L, and 8.81 µg EE2/L treatments, respectively. Abnormal male animals were under-represented in control ($p<0.01$) and solvent control ($p<0.01$) treatments and over-represented in the 0.09 µg EE2/L ($p<0.01$) and 0.84 µg EE2/L ($p<0.05$) treatments. Mixed sex animals accounted for 0%, 0%, 11%, 20%, and 33% of total genetic males in the control, solvent control, 0.09 µg EE2/L, 0.84 µg EE2/L, and 8.81 µg EE2/L treatments, respectively. Mixed sex animals were over-represented in the 8.81 µg EE2/L treatment ($p<0.01$). Phenotypic females accounted for 0%, 0%, 0%, 7%, and 17% of total genetic males in the control, solvent control, 0.09 µg EE2/L, 0.84 µg EE2/L, and 8.81 µg EE2/L treatments, respectively. Statistically, females were over-represented in the 8.81 µg EE2/L treatment ($p<0.05$).

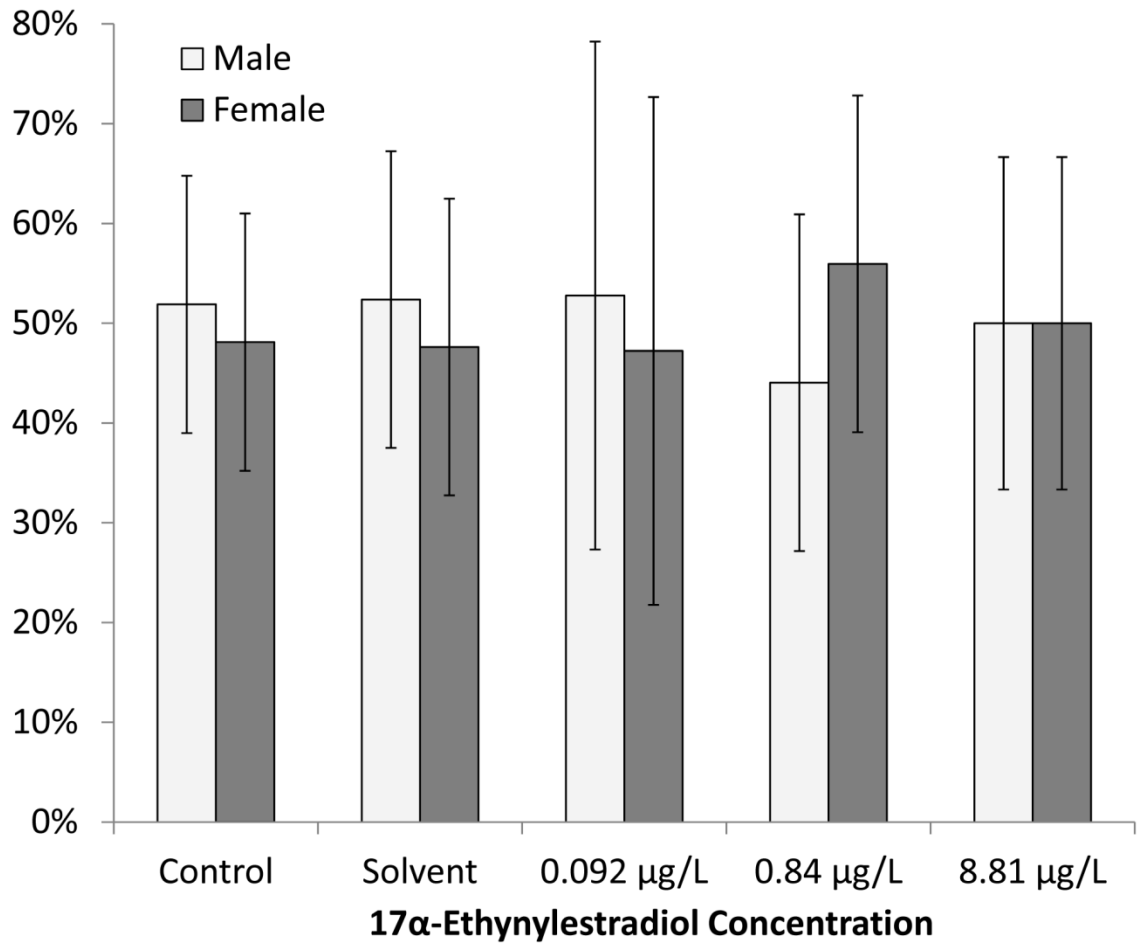

Figure 2.3: Genetic sex ratios of *Xenopus laevis* exposed to 17α-ethynylestradiol during larval development. The percentage of animals with male and female genotypes, expressed as mean ± S.D., in each treatment is shown (*n*=3 replicate tanks per treatment). The sex ratios among treatments did not differ significantly from one another (Pearson Chi-Square, p=0.860), nor did any individual treatment differ from a 50:50 sex ratio.

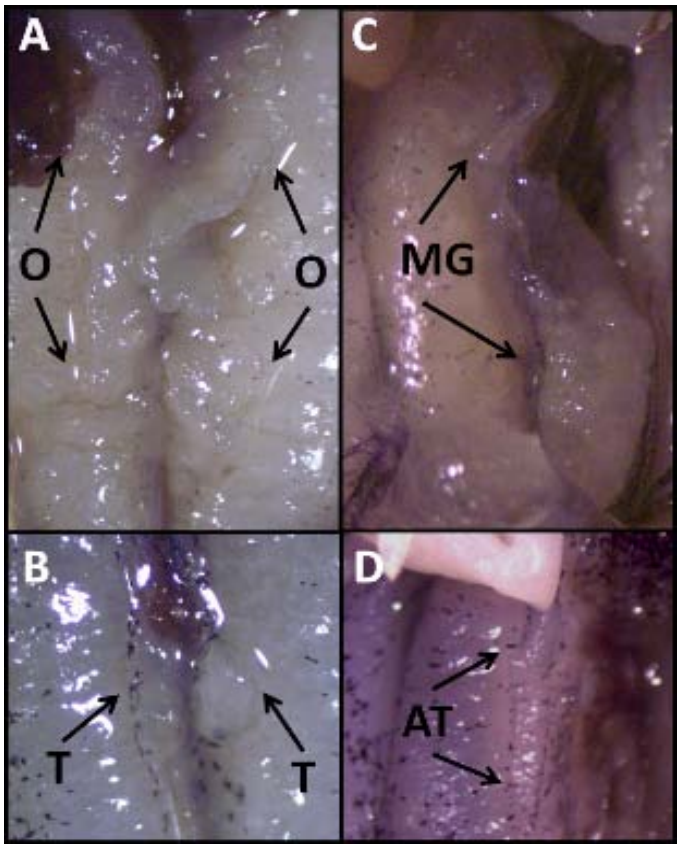

Figure 2.4: Gross morphological phenotypes of *Xenopus laevis* exposed to 17α-ethynylestradiol during larval development. Representative morphological images of the 4 phenotype classes utilized in the current study: A) Female individual with two ovaries (O); B) Male individual with two testes (T); C) Mixed sex individual with a mixed sex gonad (MG); D) Abnormal male animal with an abnormal testis (AT).

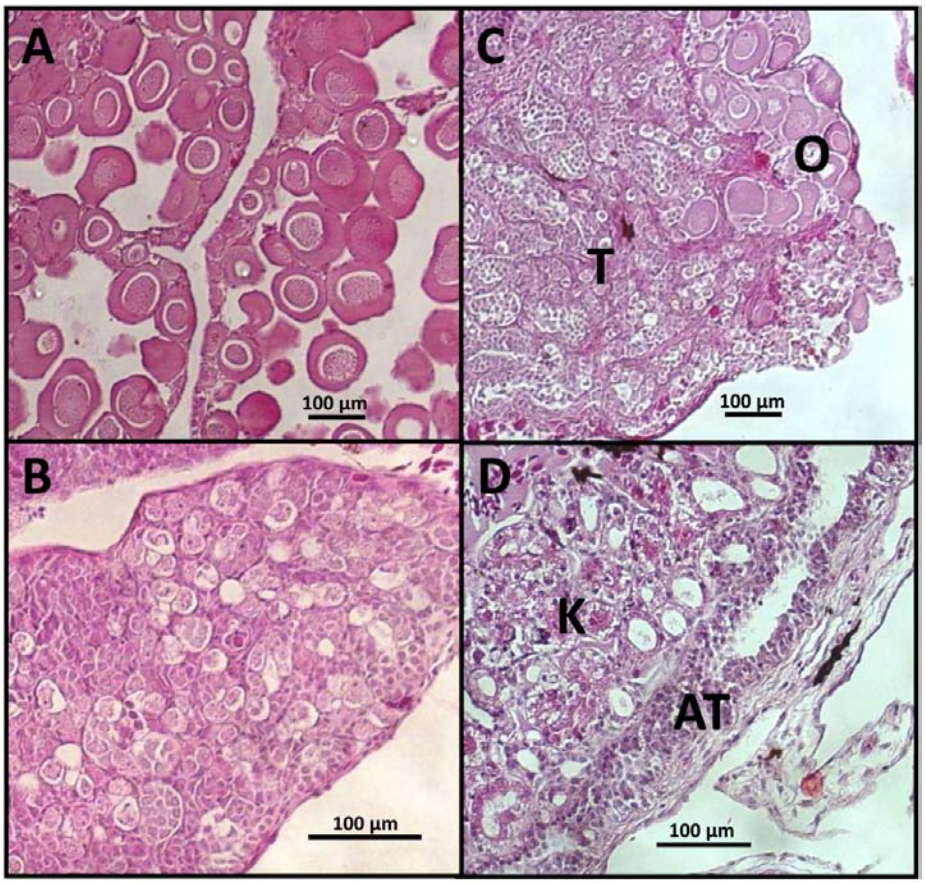

Figure 2.5: Histological phenotypes of *Xenopus laevis* exposed to 17α-ethynylestradiol during larval development. Representative histological images of the 4 phenotype classes utilized in the current study: A) Female ovary filled with primary oocytes; B) Male testis filled with spermatocysts; C) Mixed sex gonad with both testicular spermatocysts (T) and primary oocytes (O); D) Abnormal male gonad with abnormal testicular tissue (AT) lacking spermatocysts and with a central cavity. In this image the abnormal testis (AT) is shown along with kidney (K) tissue. These histological images (A-D) correspond to the morphological images presented in Figure 2.4 (i.e. image A in both figures is from the same individual).

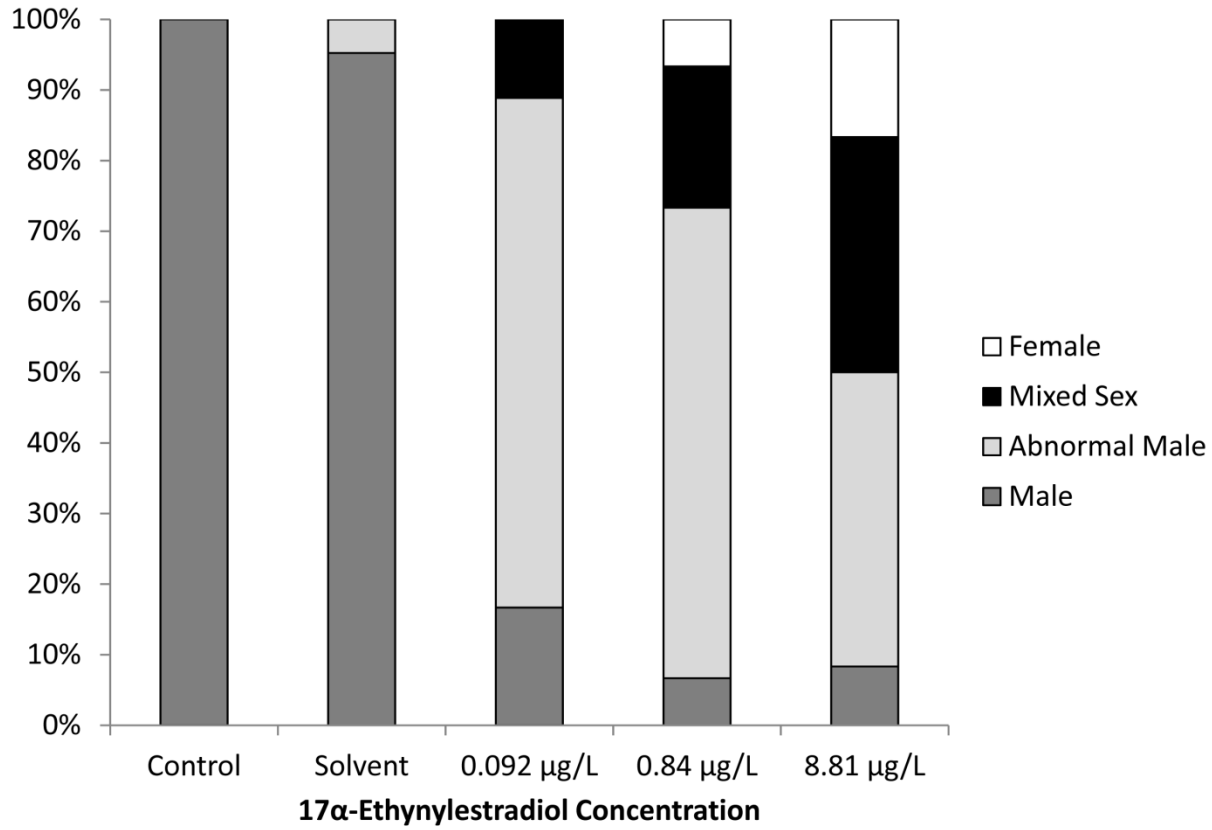

Figure 2.6: Phenotypic sex ratios of genetic male *Xenopus laevis* exposed to 17α-ethynylestradiol during larval development. The overall percentage of individuals in each phenotype category (male, female, abnormal male, mixed sex) among individuals with a male genotype (*n*=12-23 in each treatment group). There were significant differences between treatments in sex ratios (Fisher's Exact test, $p<0.0001$; post-hoc testing is detailed in Table 2.1). Increasing EE2 concentrations led to a greater proportion of genetic males developing with other phenotypes, including abnormal male, mixed sex, and female.

Table 2.1: Phenotype categories that contributed to significantly altered sex ratios among 17α-ethynyestradiol treatments.

	Phenotype			
EE2 Treatment	Male	Abnormal male	Mixed sex	Female
Control	+	- -	NS	NS
Solvent	+	-	NS	NS
0.09 µg/L	-	+ +	NS	NS
0.84 µg/L	-	+	NS	NS
8.81 µg/L	-	NS	+ +	+

- Significantly under-represented ($p<0.05$)
+ Significantly over-represented ($p<0.05$)
-- Significantly under-represented ($p<0.01$)
++ Significantly over-represented ($p<0.01$)
NS Not significant

2.4.5. Presence of vitellogenin protein

An IHC technique was developed to identify the unknown proteinaceous fluid present in and around the kidney-gonad complex of animals exposed to EE2. This technique identified the protein as VTG, a yolk precursor protein (Figure 2.7). Since only a limited number of individuals had appropriate histological slides remaining for analysis of the presence of the VTG protein, robust analysis was not possible. Analyses were performed on at least 3 individuals from each phenotype category from the EE2 treatments, and on at least 2 individuals of normal male or female phenotype in control groups. No attempt was made to quantify the amount of VTG present since IHC is much more reliable as a qualitative technique. Based on observations of IHC staining of sections, abnormal VTG production was observed in animals exposed to all concentrations of EE2 tested. Abnormal production included presence of excess VTG in the ovary, and presence of VTG in the testes, kidney, and in clouds around the kidney and gonad. In control animals, vitellogenic oocytes of normal females showed the presence of VTG, but it was never found in the testes, in the kidneys, or in clouds surrounding the kidney-gonad complex in either males or females.

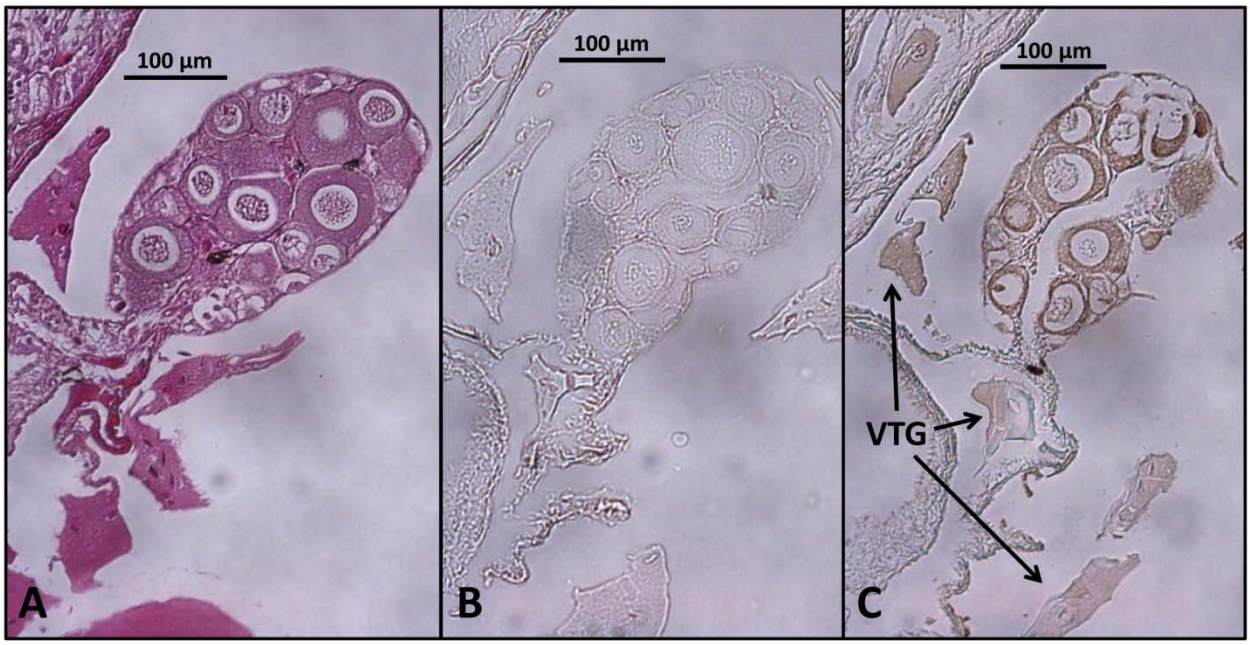

Figure 2.7: Immunohistochemical detection of vitellogenin in *Xenopus laevis* exposed to 17α-ethynylestradiol during development. Representative images from a single sex reversed individual (male genotype, female phenotype) exposed to 0.84 µg EE2/L: A) Hematoxylin and eosin stained section of ovary surrounded by clouds of proteinaceous fluid; B) Section of the ovary stained for vitellogenin immunohistochemistry (IHC) without the vitellogenin primary antibody (negative control for vitellogenin IHC); C) Section of the ovary stained for vitellogenin IHC showing presence of the vitellogenin (VTG) protein in primary oocytes within the ovary and in clouds surrounding the ovary.

2.5. Discussion

2.5.1. Effects of 17α-ethynylestradiol on sexual differentiation in *Xenopus laevis*

Previous studies of the effects of estrogens on sexual differentiation in *X. laevis* have been limited by the inability to assign a genetic sex to individual frogs or tadpoles. Using the genotyping assay developed by Yoshimoto et al. (2008), genetic sex ratios of *X. laevis* exposed to the environmentally relevant estrogen EE2 were evaluated in the current study. Genotyping allowed definitive identification of the genetic sex of individual animals, which was an improvement on previous studies where it was necessary to presume that genetic sex ratios were 50:50 to draw any conclusions about impacts of estrogens on phenotypic sex ratios. EE2 exposure was found to have greater effect on animals with a male genotype than on those with a female genotype, so it was also possible to limit statistical analyses to only those animals with a male genotype.

Prior to the current study, there was little information on the effects of EE2 on sexual differentiation of *X. laevis*. However, comparable work had been performed multiple times with E2. The *in vivo* potencies of E2 and EE2 are similar (Rankouhi et al. 2005), and previous research with E2 indicated that the doses of EE2 used in this experiment would cause a greater degree of sex reversal of *X. laevis* than was observed. In the current study, only 7% and 17% of genetic males were phenotypically sex reversed by 0.84 and 8.81 µg EE2/L, respectively. Recent studies have indicated that the EC_{50} for feminization of genetic males averages 0.2 µg E2/L (Wolf et al. 2010) and could be as little as 0.12 µg E2/L (Lutz et al. 2008). In the current study, because none of the doses tested resulted in greater than 50% sex reversal of genetic males, it was not even possible to calculate an EC_{50} value. Thus, the results of the current study indicate that the effective dose for male to female sex reversal for EE2 might be greater than those reported in previous studies for E2.

Differences in experimental design could partially explain the relatively great effective dose of EE2 required to cause reversal of sex in the current experiment. Two of the previous studies using E2 as a test chemical used a flow-though system with continuous E2 renewal (Lutz et al. 2008, Wolf et al. 2010), while a 24 hr static renewal was employed in the current study. Concentrations of EE2 in the current study did decrease by 20-43% between 24 hr water

renewals due to degradation and/or biotransformation of EE2, which would have lowered the effective dose for all treatments compared to a continuous flow-through scenario. Even accounting for this reduction, there was still a lesser degree of sex reversal in the current study than expected (Hu et al. 2008, Lutz et al. 2008, Wolf et al. 2010).

While exposure to potent exogenous estrogens affects sexual differentiation, the role of endogenous estrogens in sexual differentiation of *X. laevis* is unclear. Larvae are capable of biotransforming exogenous steroid hormones, including E2, as early as NF stage 25 (Rao et al. 1968), which is well before the time of sexual differentiation. Exposure to relatively great concentrations of exogenous estrogens betweens NF stages 50-52 feminizes/demasculinizes sexual differentiation, but *in vivo* treatment of *X. laevis* larvae with an aromatase inhibitor to decrease physiological concentrations of estrogens during this time does not masculinize the larvae (Miyata et al. 1999). This indicates that synthesis of endogenous E2 from androgens via aromatization is not necessary for normal differentiation of sexual characteristics in female *X. laevis*. In fact, concentrations of all the steroid hormones tend to be at their greatest, probably due to maternal transfer, very early in development, and sexually dimorphic expression of the sex hormones is significant only after NF stage 62, which is well past the point of sexual differentiation (Bogi et al. 2002).

Regardless of the role of endogenous production of E2 on primary sexual differentiation in genetic females, the results of previous studies have demonstrated that exposure to exogenous estrogens can affect sexual differentiation of genetic males. Since endogenous E2 doesn't seem to be integral to development of females, it is probable that the function of E2 in reversal of phenotypic males to phenotypic females is an inhibitory one. Normally, near the time of sexual differentiation, the primordial germ cells (PGCs) of animals with a male genotype would begin to migrate from the cortex to the medulla of the primordial gonad. Treatment with E2 inhibits migration of PGCs in a dose-dependent manner (Hu et al. 2008). Thus, reversal of phenotypic sex is not a matter of feminization via estrogenic effects but of demasculinization due to inhibition of normal developmental processes by estrogen. In this case, the fact that aromatase inhibitors do not cause masculinization (Miyata et al. 1999) is moot since the estrogen short-circuits a natural process unique to males. As such, estrogen doesn't appear to be absolutely necessary for the process of ovarian differentiation in genetic females, but can induce demasculinizing effects on PGCs in genetic males because tadpoles at this stage of development

do not normally express great concentrations of endogenous hormones but do have the ability (i.e. receptors) to respond to exposure to hormones.

There are a number of other species of amphibians (and fish) that exhibit dysfunctions of sexual differentiation after exposure to potent estrogens. In *X. laevis,* normal sexual differentiation involves interplay between the sex-linked gene/transcription factor *DM-W* and its gene targets. Exposure to estrogen subverts normal physiological processes in genetic males, which causes abnormal sexual differentiation. However, it is unlikely that the same exact mechanism is functioning in all species that are sensitive to exposure to estrogens. In some cases, the affected species have a different chromosome complement (XX/XY vs. ZZ/ZW), and thus dissimilar systems of sex determination. In other cases, it is clear that the species are different in some fundamental way even though the chromosome complement is the same as *X. laevis*. For example, in addition to being sensitive to feminization of males by estrogens, *Silurana (Xenopus) tropicalis* female gonadal differentiation is masculinized by exposure to inhibitors of aromatase, which would result in lesser physiological concentrations of E2 (Olmstead et al. 2009, Duarte-Guterman et al. 2010), while *X. laevis* gonadal differentiation is not (Miyata et al. 1999). This result indicates that the two species probably differ in the mechanism of determination of phenotypic sex, at least for genetic females, and in sensitivity to alterations in homeostasis of the sex steroids. Altogether, within the Anurans, the processes of sexual determination, differentiation, and development are clearly complex and differ from species to species (reviewed by Hayes 1998).

2.5.2. Effects of 17α-ethynylestradiol on metamorphosis and post-metamorphic endpoints

In the current study, exposure to EE2 delayed metamorphosis by 15-23 d in a dose-dependent manner. Delays in reaching metamorphosis after exposure to potent estrogens have been observed previously in various species, including the northern leopard frog (*Rana pipiens*) (Hogan et al. 2008), *Rana temporaria* (Roth 1948), and *X. laevis* (Richards and Nace 1978, Gray and Janssens 1990, Lutz et al. 2008). However, the mechanism by which estrogenic substances inhibit larval development is not clear. *In vitro* studies have indicated that there might be crosstalk between the estrogen receptor and the thyroid hormone receptor (Vasudevan et al.

2001). The normal responsiveness of amphibian larvae to thyroid hormones, especially triiodothyronine (T_3), is affected by *in vivo* exposure to estrogen (Hogan et al. 2008). Since thyroid hormones are responsible for orchestrating metamorphosis (Shi 2000), alterations in thyroid hormone homeostasis would likely impact metamorphic endpoints. The status of the hypothalamic-pituitary-thyroid axis was not monitored in the current study, so it is uncertain whether alterations of the thyroid hormones and receptors were induced by EE2 exposure.

Delays in reaching metamorphosis are most likely linked to alterations in thyroid hormone homeostasis, but these alterations do not necessarily have to be the result of crosstalk between the hypothalamic-pituitary-thyroid axis and the hypothalamic-pituitary-gonad axis, or their respective receptors. VTG, a yolk-precursor protein, is produced in response to exposure to estrogens, even in genetic males, via regulation by an estrogen response element in the promoter region of the gene (reviewed by Rotchell and Ostrander 2003). Early larval stages of *X. laevis* lack the ability to respond to estrogenic chemicals in this way as evidenced by their inability to produce VTG until around the time of metamorphic climax (NF Stages 60-64) (Tata et al. 1993). In the current study, expression of VTG was not evaluated at NF Stages 60-64, but analysis of post-metamorphic individuals (NF Stage 65+) revealed an apparent induction of production of the VTG protein. If these animals were also producing VTG around the time of metamorphic climax, it is possible that abnormal production of this phospholipidprotein could have contributed to the observed delayed metamorphosis. It is known that protein synthesis is an energetically expensive process, and can account for nearly 80% of cellular oxygen consumption in isolated rainbow trout hepatocytes (Pannevis and Houlihan 1992). Thus, intensive production of VTG protein in affected *X. laevis* individuals could have possibly exhausted energy stores needed for completion of metamorphosis. However, it would be necessary to perform further experiments on exposed animals near the time of metamorphic climax to validate this hypothesis.

In the current study, induction of production of VTG might have been partially responsible for differences observed between gross morphological phenotypic sex ratios and histological phenotypic sex ratios. In some cases, structures that were identified as “ovaries” upon gross morphological evaluation were just large deposits of VTG with no primary ovarian structures at the histological level. In some males exposed to EE2 the VTG protein was sequestered into pouch-like structures on top of the kidney that could easily be mistaken for an ovary. However, there was no ovarian structure present, and the pouch often obscured a normal

or abnormal testis. When phenotypic sex ratios were corrected, there was up to 42% less phenotypic sex reversal of genetic males than had been expected based upon gross morphological examination alone.

Gross morphological phenotypic evaluation was determined to be a less effective method for determination of phenotypic sex than histological evaluation in the current study. This could partially account for the difference in effective dose for sex reversal between the current study and the study by Lutz et al. (2008) which determined the EC_{50} for sex reversal of *X. laevis* to be 0.12 µg E2/L. That study used only gross morphological characterization of the gonads of frogs to assign phenotypic sex. In the current study, gross morphology was found to be extremely misleading in some cases, and histological evaluation of the gonads significantly impacted the results of phenotypic classification. Thus, in studies of this type, it is best to use the more sensitive endpoint of histological evaluation of the gonads. This recommendation was also given by some of the authors of the Lutz et al. study in a related later manuscript (Wolf et al. 2010).

2.5.3. Conclusions

Environmental exposure to potent estrogens is currently a concern for amphibians and other aquatic species. Model estrogen exposures are useful for determining effective concentrations for adverse effects on sexual differentiation and development, and the mechanisms that drive those effects. The findings of the current study indicate that studies of sex reversal can produce more useful results if performed on species that have a known sex-linked gene because it allows more robust analyses. Since different species of amphibian differ in their sensitivity to hormone exposure and sex-determining mechanisms, research directed toward discovery of sex-linked genes in amphibians other than *X. laevis* would be useful. In addition, in amphibian research, it is common to conduct experiments, including sex reversal experiments, over the entire larval period and even beyond metamorphosis. While this allows inclusion of the biologically relevant endpoint of time to metamorphosis, the current study illustrates that effects of exposure to estrogen after sexual differentiation, like induction of the synthesis of VTG, can be temporally separated from the effects of estrogen on sexual differentiation.

CHAPTER 3

Characterization of transcriptional responses of genetic male *Xenopus laevis* tadpoles exposed to 17α-ethynylestradiol during sexual determination and differentiation

3.1. Abstract

Genetic male African clawed frogs (*Xenopus laevis*) display feminized or demasculinized phenotypes after exposure to potent estrogens during the period of sexual determination and differentiation, but little is known about the molecular changes that drive the development of the altered phenotypes. Thus, the transcriptome-level effects of exposure to 17α-ethynylestradiol (EE2) at Nieukwoop-Faber Stage 53, which is during the sensitive period of sexual differentiation, were evaluated in *X. laevis* with a male genotype by use of *Illumina* sequencing coupled with RNA-Seq expression analysis. Overall, a number of pathways and processes were impacted by exposure to 17α-ethynylestradiol, including steroid and xenobiotic signaling and metabolism, steroid biosynthesis, thyroid hormone signaling and metabolism, and testicular development and spermatogenesis. At the time of the transcriptome analysis, there were no effects of treatment with EE2 on mortality or growth of *X. laevis* tadpoles. However, some of the pathways that were altered in these tadpoles, such as thyroid hormone signaling and testicular development, could be linked with observed biological effects on metamorphosis and gonadal phenotypes, respectively, which were observed in a group of frogs that was exposed to 17α-ethynylestradiol throughout larval development (89 d). Thus, the observed effects on the transcriptome predicted biological effects that did not manifest until later in development.

3.2. Introduction

Some species of fish and amphibians display phenotypic plasticity during larval development and can be feminized and/or demasculinized by exposure to estrogenic chemicals immediately prior to and during the period of sexual determination and differentiation (Chang and Witschi 1955, Witschi et al. 1958, Villalpando and Merchant-Larios 1990, Lange et al. 2001, Bogi et al. 2003, Fenske 2005, Pettersson et al. 2006). Feminization/demasculinization of amphibians and fish has the potential to affect community and population-level fitness by altering sex ratios, fertility, or fecundity (Kidd et al. 2007, Gyllenhammar et al. 2008, Hu et al. 2008). Since estrogenic substances, such as 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), enter the aquatic environment through various sources, including the discharge of liquid effluents

from wastewater treatment facilities (Ankley et al. 2007) and runoff containing animal manure (Hanselman et al. 2003), there is the potential for exposure of aquatic wildlife to these compounds during the sensitive period of sexual determination and differentiation.

The African clawed frog (*Xenopus laevis*), a commonly used model species in studies of development, has been shown to be sensitive to exposure to estrogens during sexual determination and differentiation (Chang and Witschi 1955, Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Lutz et al. 2008). Between Nieuwkoop-Faber (NF) stages 50 and 54, exposure to concentrations of E2 in the lesser µg/L range causes disordered sexual development and even reversal of the male phenotype of genetic males to a female phenotype (Villalpando and Merchant-Larios 1990, Wolf et al. 2010). Since the process of sexual differentiation is taking place during this period, the genes involved in differentiation are presumably also being expressed at this time.

The cascade of genes expressed during sexual determination and differentiation of genetically female *X. laevis* has been partially deduced. *X. laevis* has a ZW system of sex determination where females are the heterogametic sex (ZW karyotype) and males are the homogametic sex (ZZ karyotype). The Z and W chromosomes are morphologically indistinguishable (Schmid and Steinlein 1991), but recently, a sex-linked gene that is located on the W chromosome, called *DM-W*, was discovered (Yoshimoto et al. 2008). The role of this gene in female sexual development is not completely clear, but it is likely a transcription factor with DM-type DNA binding domain (Yoshimoto et al. 2010) that is expressed transiently in *X. laevis* during sexual determination, which takes place at NF stage 50 (Yoshimoto et al. 2008). The DNA binding domain of *DM-W* is 89% identical to that of the known testis determining factor *DMRT1* (Yoshimoto et al. 2008), and the two genes are thought to be paralogs (Okada et al. 2009). Evidence suggests that the *DM-W* transcription factor binds to the same genes as *DMRT1*, with *DM-W* possibly antagonizing the effects of *DMRT1* (Yoshimoto et al. 2010). In addition to antagonizing *DMRT1, DM-W* might also be capable of activating genes that are necessary for development of ovaries in females. *In vivo* transfection of genetic male tadpoles with a *DM-W* expression vector induces transcription of aromatase (*cyp19a*) and *foxl2*, a transcription factor thought to up-regulate transcription of *cyp19a*, from NF stages 50-57 (Okada et al. 2009). This pattern of *cyp19a/foxl2* expression is normally seen in genetic females but not genetic males, which indicates that these genes might be involved in primary differentiation of

ovaries (Okada et al. 2009). However, transfection of genetic male tadpoles with a *DM-W* vector is not capable of causing complete reversal of phenotypic sex, although it can induce formation of some primary ovarian structures (Yoshimoto et al. 2008). Since *DM-W* is not capable of causing reversal of the phenotypic sex of genetic male tadpoles, there must be some other factor, either W-linked or specific to the ZZ chromosome complement, which influences primary sexual differentiation in genetic female *X. laevis* (Yoshimoto et al. 2008).

It is unlikely that the gene cascade involved in normal development of ovaries in female *X. laevis* is involved in the male-to-female reversal of phenotypic sex that can be induced by exposure to estrogens. Genetic male *X. laevis* cannot express *DM-W* since they do not have a copy of the gene, and expression of *CYP19a* and *FOXL2* would be unlikely to have an effect in a system with excess estrogens, since these two genes work in concert to increase physiological concentrations of circulating estrogens. There is evidence that estrogens cause phenotypic reversal of sex due to an inhibition of migration of the primordial germ cells of genetic male animals from the cortex to the medulla of the primordial gonad (Hu et al. 2008), but there has never been an effort to determine the molecular changes responsible for this inhibited migration.

Although *DM-W* is not involved in the process of estrogen-induced male-to-female reversal of phenotypic sex, the presence of the *DM-W* gene in an individual's genomic DNA complement can be used as a marker of genetic sex. This is especially useful in cases where phenotypic sex has been reversed by exposure to estrogens or at times before the gonad has differentiated. While it becomes possible to determine phenotypic sex by use of morphology after NF stage 56 (Bogi et al. 2002), estrogen-induced reversal of phenotypic sex is indistinguishable from normal female development. Previously, to identify sex-reversed genetic males, *X. laevis* had to be grown to reproductive maturity, spawned, and the sex ratios of their offspring evaluated (Chang and Witschi 1955, Chang and Witschi 1956). Therefore, discovery of *DM-W* and subsequent creation of a genotyping assay for *X. laevis* (Yoshimoto et al. 2008) affords researchers the opportunity to perform experiments that alter sexual differentiation in *X. laevis* with the ability to draw conclusions on the basis of genetic sex at any point during development, including during the larval stages.

Recent advances in next generation sequencing technologies have made analysis of gene expression of entire transcriptomes both achievable and economically feasible. Parallel sequencing technologies, such as *Illumina's* polymerase-based sequence by synthesis or Roche's

454 pyrosequencing, coupled with software to construct and evaluate entire transcriptomes have made it possible to create "digital microarrays" that have greater specificity and dynamic range than traditional microarrays (Garcia-Reyero and Perkins 2011). Although these sequencing technologies are now financially accessible to some laboratories, their application to the field of ecotoxicology is in its infancy. Ecotoxicology experiments often have designs that complement the use of transcriptome sequencing coupled with an RNA-Seq expression analysis (reviewed by Chen et al. 2011). This type of analysis gives researchers the opportunity to compare transcriptomes of exposed and unexposed populations, either in controlled lab studies or in the field. This top-down approach is useful for discovering novel and essential biological responses without prior knowledge of these responses and could ultimately be used to form definitive linkages between molecular responses and effects on individuals or populations (Ankley et al. 2009, Ankley et al. 2010, Garcia-Reyero and Perkins 2011). Thus, application of these technologies to genetic male tadpoles that have been exposed to estrogens during sexual determination and differentiation could be used to determine the molecular changes that are responsible for the observed effects of estrogen on the development of the gonads.

Both E2 and its synthetic analog EE2 are considered environmentally relevant chemicals of concern due to their presence in effluents from wastewater treatment plants (Ankley et al. 2007). Much of the previous work performed with *X. laevis* has used E2 as a test chemical, so in the current study EE2 was used as a model chemical to expand knowledge of the response of *X. laevis* to different potent estrogens. EE2 is the synthetic estrogen used in human oral contraceptives and is as much as two times as potent as E2 *in vitro* (Folmar et al. 2002) and twice as persistent as E2 in the environment (Ying et al. 2002). Thus, it was predicted that if concentrations of EE2 equivalent to E2 concentrations that caused effect were used that biological effects would be similar or greater. Based on results previously published in the literature, the nominal concentrations of EE2 chosen for the current study, 0.1, 1, and 10 µg EE2/L, were expected to cause few, moderate, and severe effects (complete reversal of male phenotypes to female phenotypes), respectively. The concentrations chosen for the current study were based upon their ability to elicit effects on phenotype, but the least dose was only about 3-fold greater than the range of estrogen equivalents (about 5-30 ng/L) that would be expected in a natural watershed (reviewed by Kidd et al. 2007).

The current study was designed to elucidate the molecular mechanisms associated with alterations in the sexual differentiation and development of *X. laevis* with a male genotype exposed to the model estrogen EE2. To do so, samples of total RNA from both exposed and unexposed male tadpoles at NF stage 53 of development (during sexual differentiation) were sequenced with an *Illumina* sequencer. After sequencing, the transcriptomic data were evaluated using an RNA-Seq expression analysis to compare the transcriptomes of tadpoles that were exposed to EE2 to those that were not exposed to EE2. To determine the possible biological relevance of changes at the transcriptomic level, the data were also evaluated in context with biological data, which has been previously reported, that was collected from a group of *X. laevis* tadpoles that were grown through metamorphosis (89 d) in water containing EE2 (Tompsett et al. 2012).

3.3. Materials and methods

3.3.1. *Xenopus laevis*

Prior to commencement of research, approval for the use of animals and all experimental procedures was obtained from the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan (Animal Use Protocol #20090066). Sexually-mature, adult *X. laevis* were purchased from Boreal Laboratories (St. Catharine's, ON, Canada) and acclimated to laboratory conditions (18±2 °C water temperature; 12:12 light-dark cycle; fed Nasco frog brittle [medium nuggets] (Salida, CA, USA) *ad libitum* daily) for one month. *X. laevis* were bred and eggs were collected as has been previously described (Tompsett et al. 2012).

3.3.2. 17*α*-ethynylestradiol exposure

Healthy fertilized eggs (50 per tank) were placed into 6L of FETAX with the appropriate nominal concentration of EE2 (0.1, 1, or 10 µg/L; Sigma) dissolved in an ethanol carrier

(Commercial Alcohols 95% ethyl alcohol, Toronto, ON, Canada) at about 13 hrs post-oviposition. The final concentration of ethanol in treatment tanks, including solvent control tanks, was 0.0025%. All treatments were replicated in triplicate tanks. The percentage of eggs that hatched in each tank was recorded. Feeding, water renewals, and basic water quality measurements were performed as has been previously described (Tompsett et al. 2012),

The average tadpole reached NF stage 53 after 31 d of exposure. At this time, 2-4 tadpoles were removed from each tank and euthanized in an overdose of MS-222 (Sigma). Differing numbers of tadpoles were taken from each tank due to slight differences in developmental stage between individuals and differences in the number of tadpoles per tank due to differing percent hatch values and tadpole mortalities. Only individuals that could be definitely identified as NF stage 53 individuals were sampled. Of the remaining tadpoles, about 30 per tank were removed later during larval development for analysis of growth and development that is not presented here. After removal of these samples, the 15 remaining tadpoles were allowed to complete metamorphosis and were subjected to analyses that have been reported previously (Tompsett et al. 2012).

After euthanasia, each NF stage 53 tadpole was weighed and measured. Then, the body and tail of the tadpole were separated for subsequent analyses. The tail portion of the tadpole was preserved at -20 °C for use in an assay to determine genetic sex. The body portion of the tadpole was flash frozen in liquid nitrogen and stored at -80 °C for subsequent molecular analyses. Only tadpoles with a male genotype were subjected to these molecular analyses since estrogen-induced feminization/demasculinization during sexual differentiation does not take place in genetic females.

3.3.3. Analysis of 17α-ethynylestradiol concentrations

Concentrations of EE2 in exposure water were monitored weekly during the experiment via high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) to validate that expected nominal values were approximated. EE2 was quantified following methods described elsewhere (Tompsett et al. 2012, Chang et al. 2010). Briefly, samples of whole water were spiked with a deuterated internal EE2 standard (C/D/N Isotopes, Inc., Pointe-

Claire, QC, Canada), extracted two times with hexane and then concentrated under nitrogen. Dried organic extracts were derivatized with dansyl chloride, re-extracted with hexane, dried under nitrogen, and then reconstituted in acetonitrile. Analytical detection was conducted using an Agilent 1200 series HPLC system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole MS/MS system (PE Sciex, Concord, ON, Canada). Both the LC and the mass spectrometer were controlled by AB Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA, USA).

3.3.4. Determination of genetic sex

In *X. laevis, t*he *DM-W* gene can be used as a marker of female genetic sex, and its absence is a marker of male genetic sex. A multiplex PCR assay has been developed to determine genetic sex in *X. laevis* individuals (Yoshimoto et al. 2008). This assay was performed as has been described previously (Tompsett et al. 2012) on genomic DNA samples extracted from the tails of NF stage 53 tadpoles. Briefly, the multiplex PCR assay included primers to amplify a fragment of the *DM-W* gene as well primers to amplify a fragment of an autosomal gene. Individuals whose genomic DNA amplified both *DMRT1* and *DM-W* were classified as genetic females while individuals that only amplified *DMRT1* were classified as genetic males. Only genetic males were subjected to further molecular analyses.

3.3.5. RNA Isolation

Total RNA was isolated from NF stage 53 *X. laevis* tadpoles with a male genotype by use of an RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was checked on a 1% denaturing formaldehyde-agarose gel with ethidium bromide and visualized under ultraviolet light on a VersaDoc 4000MP imaging system (BioRad, Mississauga, ON, Canada). Samples of purified RNA were stored at -80°C until further analyses were performed.

3.3.6. Complimentary DNA Library Preparation, *Illumina* Sequencing, and RNA-Seq

Two tadpole RNA samples were sequenced on an *Illumina* Hi-Seq 2000 Sequencer (*Illumina*, San Diego, CA, USA). Each sample consisted of 5 µg of RNA from 3 individuals that was pooled into a 15 µg RNA sample. One sample consisted of pooled RNA from 3 individuals from the solvent control treatment, and the other RNA sample consisted of pooled RNA from 3 individuals from the 1 µg EE2/L treatment. The integrity of the samples of RNA was evaluated with a 2100 Bioanalyzer (Agilent, Clara, CA, USA), then they were prepared and sequenced by the National Research Council of Canada Plant Biotechnology Institute (Saskatoon, SK, Canada). Briefly, cDNA libraries were prepared using an mRNA-Seq Sample Prep Kit (*Illumina*) according to the manufacturer's protocol. Libraries were then sequenced as 2x100bp paired-end reads on a Hi-Seq 2000 Sequencer (*Illumina*). Each pooled sample was sequenced in an individual lane to maximize the number of reads per sample.

Individual files containing sequence information from the *Illumina* sequencing run were imported into CLC Genomics Workbench V 5.1 software (CLC Bio, Aarhus, Denmark) for each RNA sample sequenced, and subsequent analyses, unless otherwise noted, were performed using the CLC Genomics Workbench. Sequences were trimmed of adapter sequences and ambiguous nucleotides and filtered for quality using a modified-Mott trimming algorithm. Reads with lengths of less than 30 nucleotides were discarded. For each sample of RNA, sequences that met quality criteria were then mapped to a reference transcriptome by use of RNA-Seq with individual gene expression values normalized as reads per kilobase of exon model per million mapped reads (RPKM) (Mortazavi et al. 2008). The reference transcriptome consisted of all 11,654 full-length *X. laevis* cDNAs currently available in the National Centre for Biotechnology Information sequence database (http://www.ncbi.nlm.nih.gov). Once the RNA-Seq had been performed for each sample, an expression analysis was performed to compare expression values for the two RNA samples to one another. Reference transcripts with fewer than 10 mapped gene reads in at least one sample of RNA were eliminated from the expression analysis. Fold-change values were calculated based upon RPKM and a significant change in the abundance of a transcript was defined as a fold-change value greater than or equal to ±2-fold. The ±2-fold significance level was a chosen because it is a conservative value for determining both statistical

and biological differences in abundances of transcripts according to previous studies (Brooks et al. 2011, Jung et al. 2011, Glaus et al. 2012)

Separate lists of transcripts that were up-regulated or down-regulated at least ±2-fold were compiled into FASTA files. These transcript files were imported into Blast2GO software (Cosena et al. 2005, Götz et al. 2008) and the identity and gene ontology (GO) of each transcript was determined. The pathways impacted by exposure to EE2 were determined by analysis of both the GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) enzyme mapping using Blast2GO.

3.3.7. Statistics

Statistical tests were performed by use of IBM SPSS Statistics 19 software (IBM, Armonk, NY). Treatment means are expressed as mean ± S.D. throughout. Statistical significance was defined as $p \leq 0.05$. Where appropriate, data were subjected to tests of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) to determine whether to apply parametric or nonparametric statistical tests. Percent hatch and mortality data were analyzed with Pearson Chi-Square tests to determine differences in proportions among treatments. The data for mass and length of tadpoles at NF stage 53 were analyzed with ANOVA or Kruskal-Wallis tests, for parametric and non-parametric data, respectively, with post-hoc Tukey's tests to determine differences among treatment means.

3.4. Results

3.4.1. Water quality and validation of 17α-ethynylestradiol concentrations

Water quality parameters over the course of the experiment were all within an acceptable range for culture of *X. laevis*. Average values were temperature of 22.6 ± 0.1 °C, conductivity of 1.70 ± 0.02 mS/cm, 7.3 ± 0.1 mg dissolved oxygen/L, pH of 7.8 ± 0.1 standard units, and 0.02 ±

0.01 mg ammonia/L. Nitrite was never detected above the method detection limit of 0.02 mg/L. Nitrate was never detected above the method detection limit of 0.25 mg/L.

The limit of quantification for the LC-MS/MS results presented for the current study was 0.02 µg EE2/L. Within this limit, EE2 was not detected at any point during the experiment in control or solvent control tanks. Nominal concentrations of EE2 treatments were 0.1, 1, and 10 µg EE2/L, and actual concentrations were 0.09 ± 0.005, 0.84 ± 0.06, and 8.81 ± 1.25 µg EE2/L immediately following water changes, respectively. To determine whether concentrations of EE2 remained stable over the 24 hr period between water renewals, concentrations were also determined just prior to water change. The $t_{1/2}$ values for EE2 were 2.8, 1.2, and 2.1 d in the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively. Since EE2 concentrations just prior to water change were not monitored as often as concentrations immediately following water change, validated concentrations of EE2 after water change are used hereafter to designate treatment groups.

3.4.2. Percent hatch and mortality of tadpoles

On average, 92% of *X. laevis* eggs survived to hatching. Within treatment, the number of eggs that hatched ranged from 90% in control water to 93% in solvent control water, 0.84 and 8.81 µg EE2/L. There were no differences among treatments in percent hatch (Pearson Chi-Square, p=0.888). Through NF stage 53, mortality of tadpoles ranged from 3% when exposed to 0.84 µg EE2/L to 5% for tadpoles exposed to 8.81 µg EE2/L. Overall average mortality was 4%. There were no differences in mortality among treatments (Pearson Chi-Square, p=0.910). Although there is no mortality guideline for the type of experimental design utilized in the current study, mortality was at or below the standard set for a valid study (≤10%) by both ASTM for the Frog Embryo Teratogenesis-*Xenopus* (FETAX) Assay (ASTM 2004) and the US Environmental Protection Agency for the 21-day Amphibian Metamorphosis Assay utilizing *X. laevis* (USEPA 2011).

3.4.3. Mass and length of tadpoles at NF stage 53

There were no significant differences in mass among treatments (Kruskal-Wallis, p=0.24; Figure 3.1). The average tadpole mass at NF stage 53 was 0.15 ± 0.01 g in control tanks, 0.18 ± 0.4 g in solvent control tanks, 0.17 ± 0.03 g in 0.09 µg EE2/L treatment tanks, 0.22 ± 0.08 g in 0.84 µg EE2/L treatment tanks, and 0.23 ± 0.05 g in 8.81 µg EE2/L treatment tanks. There were no significant differences in length among treatments (ANOVA, p=0.11; Figure 3.1). The average length of a tadpole at NF stage 53 was 29.8 ± 1.1 mm in the control treatment, 34.2 ± 2.9 mm in the solvent control treatment, 33.0 ± 2.3 mm in the 0.09 µg EE2/L treatment, 34.7 ± 4.3 mm in the 0.84 µg EE2/L treatment, and 37.2 ± 2.5 mm in the 8.81 µg EE2/L treatment.

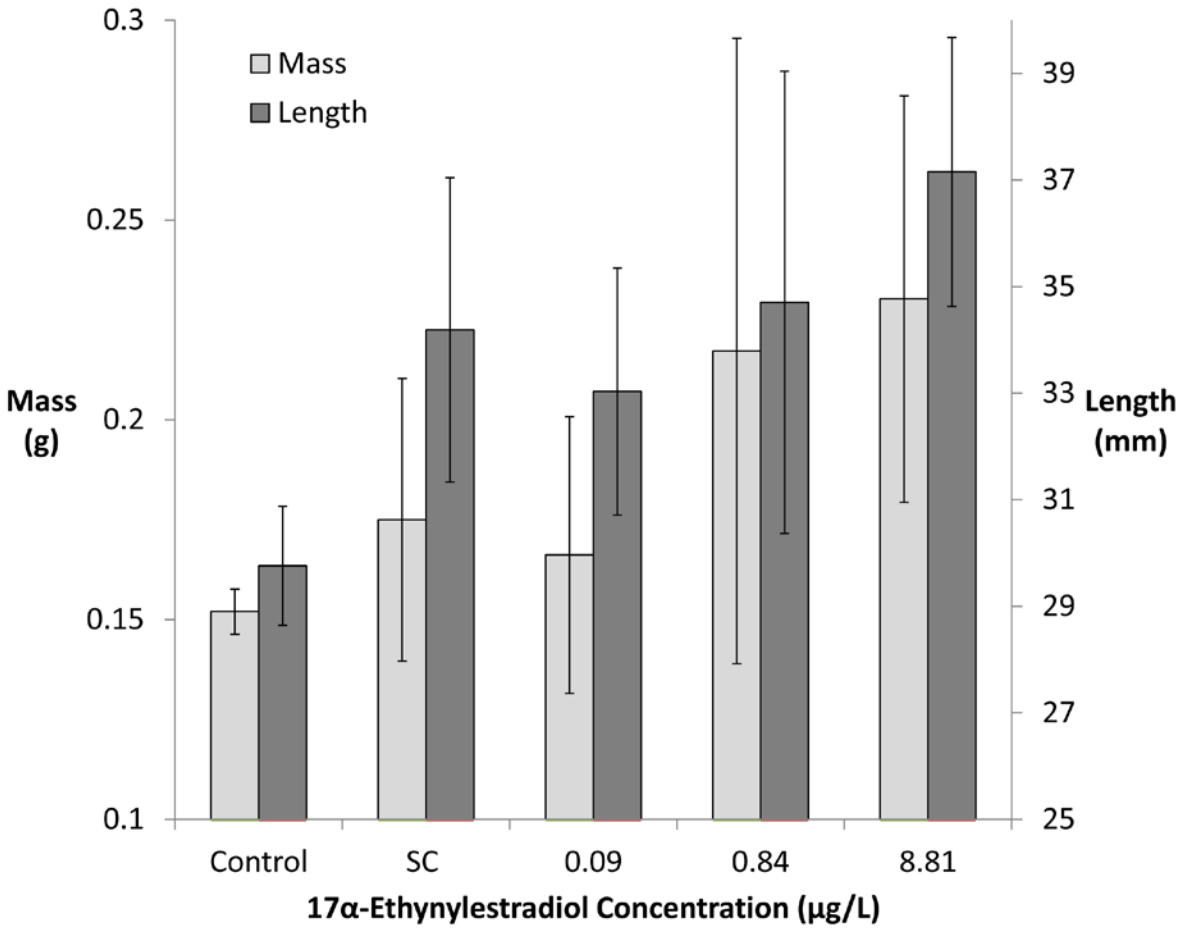

Figure 3.1: Mass and length of genetic male *Xenopus laevis* tadpoles at Nieuwkoop-Faber stage 53 after exposure to 17α-ethynylestradiol during larval development. Data are expressed as mean ± S.D (*n*=3 replicate tanks). There were no significant differences among treatments in mass (Kruskal-Wallis, p=0.24) or length (ANOVA, p=0.11)

3.4.4. *Illumina* sequencing and RNA-Seq expression analysis

A total of 72,835,794 and 91,814,234 high quality reads were sequenced for the RNA isolated from the solvent control tadpoles and tadpoles exposed to 0.84 µg EE2/L, respectively. When these reads were mapped to the reference transcriptome of 11,654 *X. laevis* cDNAs, 67% and 72% of the reads mapped to the transcriptome for the solvent control tadpoles and the tadpoles exposed to 0.84 µg EE2/L, respectively (Figure 3.2). The reads that mapped to the reference transcriptome were subjected to an analysis of expression by use of RNA-Seq to determine which transcripts from the reference transcriptome were of 2-fold greater or lesser abundance. Within this limit, abundances of transcripts of 126 genes were of greater abundance and the abundances of transcripts of 399 genes were of lesser abundance in in tadpoles exposed to 0.84 µg EE2/L, compared to those exposed to the solvent control. These figures correspond to 1.1% and 3.4% of the total number of transcripts in the transcriptome that were up- and down-regulated, respectively (Figure 3.3).

The 126 transcripts that were of 2-fold or greater abundances and the 399 transcripts that were of 2-fold or lesser abundances were subjected to GO and KEGG pathway analyses. For those transcripts that were of greater abundances, a number of processes and pathways that are either known to be affected by exposure to potent estrogens or known to be involved in sexual development were indicated. The up-regulated pathways and processes included steroid and xenobiotic signaling and metabolism, gonadal development, and biosynthesis of steroids. The individual genes involved in these pathways and processes included nuclear receptors, enzymes, and a transcription factor (for a selection of genes representative of these pathways see Table 3.1). Similarly, for those transcripts that were of lesser abundances, a number of pathways and processes that have been shown to be impacted by exposure to estrogens or that are involved in sexual development were identified. Specifically, pathways and processes related to steroid and xenobiotic signaling and metabolism, testicular development, retinoic acid signaling, thyroid hormone signaling and metabolism, biosynthesis of cholesterol, and biosynthesis of steroids were impacted. The individual genes involved in these pathways and processes included nuclear receptors, enzymes, and transcription factors (for a selection of genes representative of these pathways see Table 3.2).

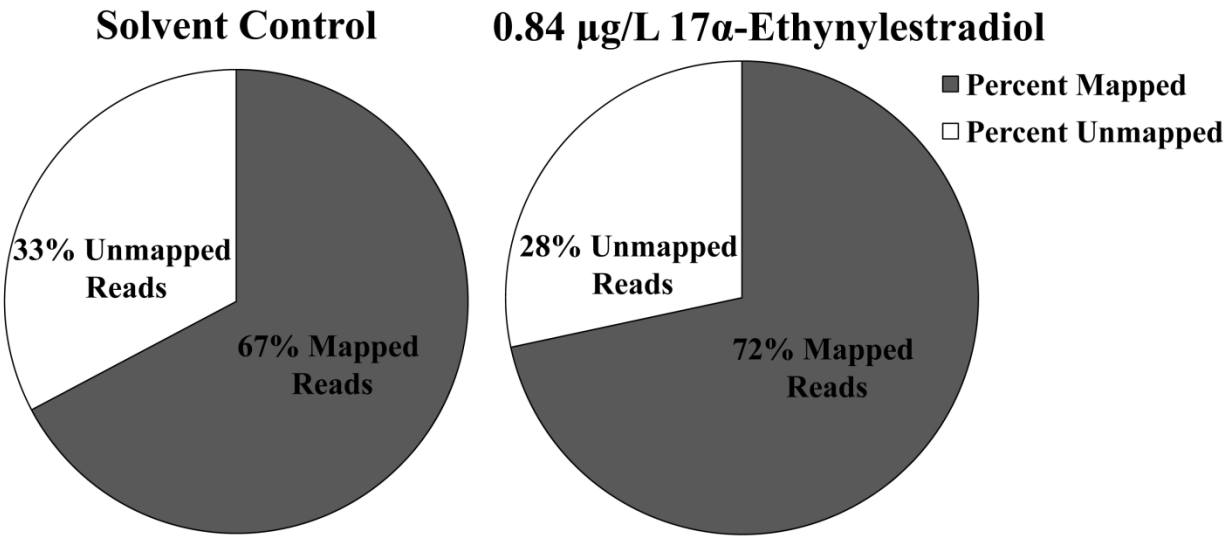

Figure 3.2: Percent of mapped and unmapped *Illumina* sequencing reads. Reads were mapped to a reference transcriptome of known *Xenopus laevis* cDNAs. Approximately 70% of reads mapped to these previously published sequences.

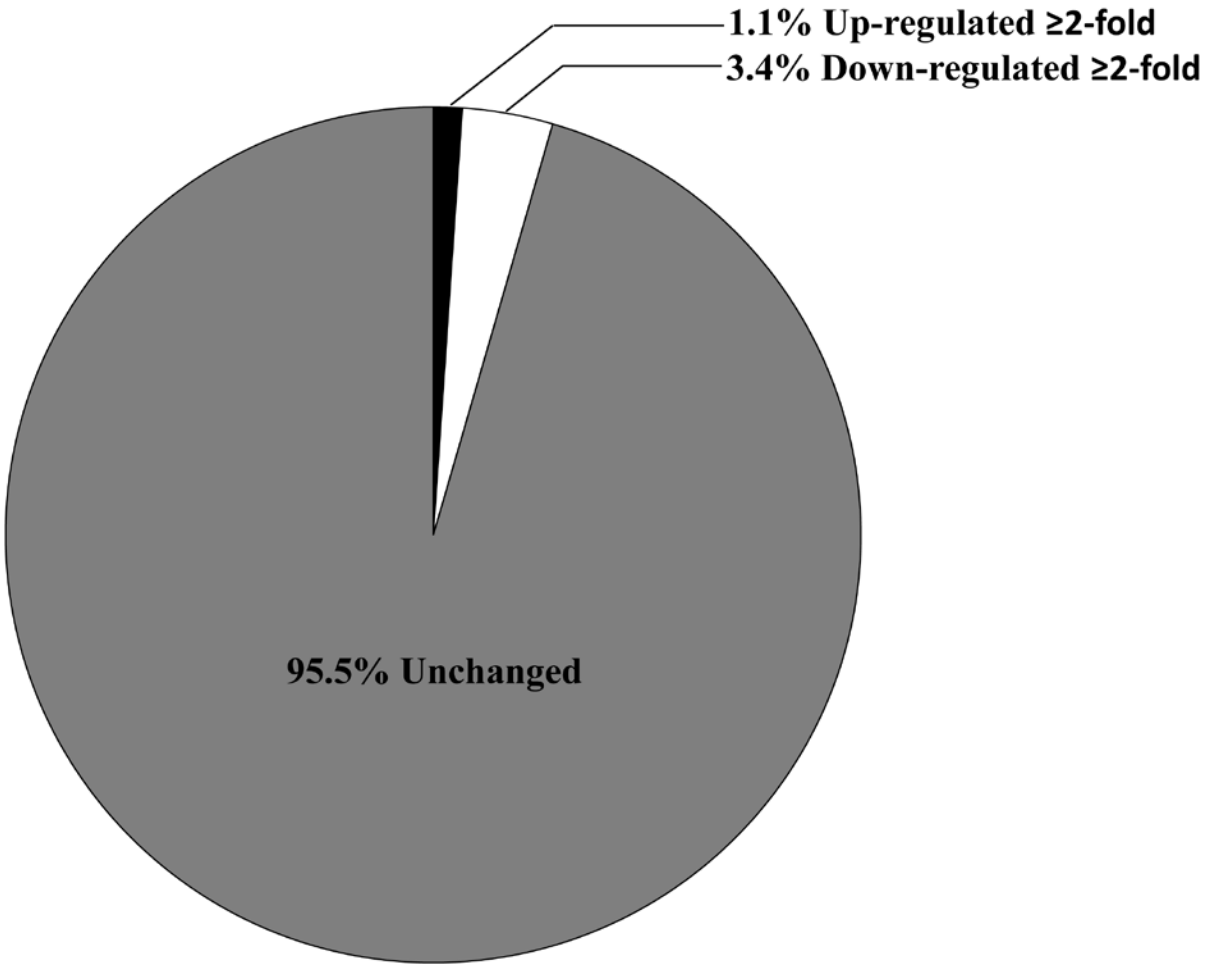

Figure 3.3: Percent of transcripts that were of greater (≥2-fold), lesser (≥2-fold) or unchanged abundance in Nieuwkoop-Faber stage 53 genetic male *Xenopus laevis* tadpoles exposed to 0.84 µg/L 17α-ethynylestradiol compared to solvent control tadpoles. Among reads that mapped to the reference transcriptome, about 4.5% were significantly altered by exposure.

Table 3.1: Selected transcripts up-regulated by at least 2-fold in genetic male *Xenopus laevis* tadpoles exposed to 0.84 µg/L 17α-ethynylestradiol utilizing RNA-Seq for expression analysis

Gene Identity	Abbreviation	Function	Fold-change[a]
3β-hydroxysteroid dehydrogenase	*3β-hsd*	SB[b]	+2.4
11β-hydroxysteroid dehydrogenase	*11β-hsd*	SB	+2.4
Cytochrome P450 2A6	*cyp2a6*	SXSM	+5.5
Nuclear receptor 1H4-like	*nr1h4-like*	SXSM	+9.5
Fushi tarazu transcription factor-1	*ftzf1*	GD	+2.7

[a] Fold-change values for RNA-Seq are normalized using the number of reads per kilobase of exon model per million mapped reads (RPKM)

[b] SB=steroid biosynthesis; SXSM=steroid and xenobiotic signaling and metabolism; GD=gonadal development

Table 3.2: Selected transcripts down-regulated by at least 2-fold in genetic male *Xenopus laevis* tadpoles exposed to 0.84 µg/L 17α-ethynylestradiol utilizing RNA-Seq for expression analysis

Gene Identity	Abbreviation	Function	Fold-change[a]
Acetyl-CoA acetyltransferase 2	*acat2*	CB[b]	-2.1
Farnesyl diphosphate synthase	*fdps*	CB	-2.3
Cytochrome P450 26A1	*cyp26a1*	RAS	-2.0
Cytochrome P450 26C1	*cyp26c1*	RAS	-2.0
Retinoic acid receptor α	*rara*	RAS	-2.2
5α-reductase	*5ar*	SB	-2.3
Aryl hydrocarbon receptor 1α	*ahr1a*	SXSM	-2.3
Cytochrome P450 2C20	*cyp2c20*	SXSM	-2.2
Cytochrome P450 2K4-like	*cyp2k4-l*	SXSM	-2.4
Glutathione-S-transferase pi-1	*gstp1*	SXSM	-2.2
Nuclear receptor 2F1	*nr2f1*	SXSM	-2.0
Short-chain dehydrogenase/reductase 42E1	*sdr42e1*	SXSM	-2.3
CUGBP, Elav-like family member 1	*celf1*	TDS	-3.3
Doublesex and mab-3 related transcription factor 1 β	*dmrt1β*	TDS	-2.2
Gametocyte specific factor 1	*gtsf1*	TDS	-2.5
Prolactin receptor α	*pra*	THSM	-2.1
Sulfotransferase 6B1	*sult6b1*	THSM	-3.7
Transthyretin	*ttr*	THSM	-2.9
Thyrotropin-releasing hormone	*trh*	THSM	-2.9
Thyrotropin-releasing hormone receptor 2	*trhr2*	THSM	-2.7
Thyroid transcription factor 1	*ttf1*	THSM	-2.2

[a] Fold-change values for RNA-Seq are normalized using the number of reads per kilobase of exon model per million mapped reads (RPKM)

[b] CB=cholesterol biosynthesis; RAS=retinoic acid signaling; SB=steroid biosynthesis; SXSM=steroid and xenobiotic signaling and metabolism; TDS=testicular development and spermatogenesis; THSM=thyroid hormone signaling and metabolism

3.5. Discussion

In the current study, transcriptome analysis, by use of *Illumina* sequencing and RNA-Seq, was utilized to determine the processes, pathways, and individual genes that were impacted by exposure of genetic male *X. laevis* to EE2, a potent estrogen, during the period of sexual determination and differentiation. To our knowledge, this is the first effort to deduce these molecular pathways in *X. laevis* using transcriptomic techniques. Since exposure to estrogens can cause abnormal sexual development and male-to-female reversal of phenotypic sex in *X. laevis* (Chang and Witschi 1955, Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Lutz et al. 2008, Wolf et al. 2010, Tompsett et al. 2012), this experiment was useful to determine the biological processes that might be involved in this abnormal development, as well as to determine responses not necessarily related to sexual development that are induced by exposures to estrogens like EE2. The use of a molecular approach was especially useful in the current study, since none of the biological endpoints monitored in tadpoles, including percent hatch, mortality, and growth endpoints, were significantly impacted by exposure to EE2.

While analyses of phenotypic sex could not be performed in the current study, the phenotypic effects of exposure to the concentrations of EE2 utilized were determined in a group of animals that was grown through metamorphosis and early post-metamorphic development (89 d) (Tompsett et al. 2012). Previous studies performed with E2 had indicated that the two greatest concentrations of EE2 utilized had the potential to cause 50-100% reversal of phenotypic sex of genetic males (Lutz et al 2008, Wolf et al. 2010), but actual values in the 89 d experiment were 7% and 17% reversal of phenotypic sex when exposed to 0.84 and 8.81 µg EE2/L (Tompsett et al. 2012). Since the genetic male tadpoles utilized for the RNA-Seq expression analysis in the current study were exposed to 0.84 µg EE2/L, it is unlikely that the individual tadpoles were undergoing complete reversal of phenotypic sex. However, the histological analyses performed on the 89 d experiment group did indicate that 83% of these animals would suffer from some degree of atypical sexual development of the gonads, including 11% of individuals developing as mixed sex and 72% of individuals developing as abnormal males (Tompsett et al. 2012). As such, the molecular changes identified by the RNA-Seq in the current study are probably not indicative of changes associated with reversal of phenotypic sex so much as with abnormal sexual development of genetic males.

3.5.1. Biological processes up-regulated in genetic male *Xenopus laevis* tadpoles

The transcriptomes of genetic male tadpoles exposed to EE2 during sexual determination and differentiation were evaluated using RNA-Seq at NF stage 53, which is after sexual determination has taken place (NF stages 50-52) and during the period of sexual differentiation (NF stages 53-59) (Nieuwkoop and Faber 1994). The RNA-Seq analysis, coupled with GO and KEGG analyses, indicated that a number of biological processes were up-regulated in individuals exposed to 0.84 µg EE2/L. These processes included steroid and xenobiotic signaling and metabolism, gonadal development, and biosynthesis of steroids (Table 3.2). The individual genes indicated within these pathways might be an indication of exactly how these processes were being impacted by exposure to EE2.

The abundances of transcripts of two genes involved in steroid and xenobiotic signaling and metabolism, *cyp2a6* and *nr1h4-like*, were greater in genetic male tadpoles exposed to EE2. The *CYP2A6* gene is involved in metabolism of nicotine, coumarin, and some pharmaceuticals, and has been shown to be inducible by exposure to E2 in human cell lines (Higashi et al. 2007). The *NR1H4-like* gene, which is also called *FOR2* in *X. laevis*, codes for an orphan nuclear receptor related to the mammalian farnesoid X receptor (*FXR*) that is thought to be involved in metabolism of endogenous and exogenous compounds and liver development (Seo et al. 2002).

Two genes involved in the biosynthesis of steroids, *3β-HSD* and *11β-HSD*, had greater abundances of transcripts in genetic male tadpoles exposed to 0.84 µg EE2/L. The *3β-HSD* gene codes for an enzyme that is important for determining the balance of synthesis of the different steroid hormones, since this enzyme can catalyze more than one reaction in the steroid biosynthetic pathway. One of the reactions catalyzed 3β-HSD produces androstenedione, the precursor to estrogens and androgens, and modeling has shown that alterations in 3β-HSD activity are likely to affect production of androstenedione (Nguyen et al. 2012). The *11β-HSD* gene codes for an enzyme that is involved in androgen synthesis in fish, and greater expression of this gene has been documented after exposure to the anti-androgen flutamide, even though the exposure phenotypically feminized the fish (Filby et al. 2007). This result, coupled with the phenotypic data collected from frogs that were exposed to EE2 for 89 d, which indicated that exposure to EE2 demasculinized or feminized genetic male *X. laevis* (Tompsett et al. 2012), indicates that greater transcription of genes involved in androgen biosynthesis does not

necessarily indicate masculinization, and paradoxically might be associated with feminization. In fact, transcriptional experiments performed on the livers of the frogs from the 89 d experiment indicated that the abundance of transcripts of the androgen receptor were greater in frogs exposed to EE2, even after 89 d (discussed in Chapter 4).

The RNA-Seq also indicated that exposure to EE2 might have affected the development of gonads in genetic males. The abundance of *ftzf1* transcripts was greater in genetic male tadpoles exposed to EE2. The *FTZF1* gene codes for a transcription factor involved in cellular differentiation and development of the germ cells in both male and female amphibians. It has been shown to be expressed in immature oocytes in *X. laevis* that were transfected with the *Rana rugosa FTZF1* gene (Asahi et al. 2002) and in the developing germ cells of both male and female *R. rugosa* (Takase et al. 2000). Thus, this gene seems to be important for development of gonads in both male and female amphibians, and its alteration might contribute to abnormal development of gonads in amphibians. However, further targeted studies are required to elucidate whether up-regulation of this gene would have any impacts on sexual differentiation of either the testis or the ovary.

3.5.2. Biological processes down-regulated in genetic male *Xenopus laevis* tadpoles

The biological processes down-regulated in NF stage 53 genetic male tadpoles exposed to EE2 during sexual differentiation included cholesterol biosynthesis, retinoic acid signaling, biosynthesis of steroids, steroid and xenobiotic signaling and metabolism, testicular development and spermatogenesis, and thyroid hormone signaling and metabolism (Table 3.3). While some of the individual genes that were impacted could be mechanistically significant, the down-regulated processes are perhaps more interesting when anchored to the phenotypic and biological data gathered from *X. laevis* individuals that were exposed to EE2 throughout larval and early post-metamorphic development (89 d) (Tompsett et al. 2012).

In the 89 d experiment, exposure to EE2 adversely impacted the testicular development of 83% of individuals exposed to 0.84 µg EE2/L (Tompsett et al. 2012). In the RNA-Seq expression analysis, three genes that are involved in the development of the testis and spermatogenesis, *celf1, dmrt1β,* and *gtsf1*, were down-regulated by at least 2-fold in genetic

males exposed to EE2. Since complete reversal of sex of genetic males was relatively rare (7%) at this concentration of EE2, it is possible that the down-regulation of these genes was at least partially responsible for the development of the majority (76%) of genetic males with abnormal and mixed sex phenotypes (Tompsett et al. 2012). This abnormal development could also have been impacted by lesser abundance of 5α-reductase (*5ar*) transcripts. The *5AR* gene codes for an enzyme that catalyzes the conversion of testosterone to dihydrotestosterone, so reductions in *5ar* transcripts might lead to lesser circulating concentrations of dihydrotestosterone. Lesser abundances of transcripts of *5ar* have previously been linked to mixed sex conditions in *Silurana (Xenopus) tropicalis* (Duarte-Guterman et al. 2010).

Based upon the abundances of transcripts indicated in the RNA-Seq analysis, the processes of thyroid hormone signaling and metabolism and retinoic acid signaling might have been down-regulated in genetic male *X. laevis* after exposure to EE2. These two pathways are known to interact via the formation of thyroid hormone receptor-retinoic acid receptor (THR-RAR) complexes (Lee and Privalsky 2005) that can induce expression of genes through a common response element (Umesono et al. 1988). Normally, *X. laevis* complete resorption of the tail and metamorphose at NF stage 66, about 6-8 weeks after hatch. Exposure to 0.84 µg EE2/L for 89 d significantly delayed metamorphosis of tadpoles into juvenile frogs by 17 d relative to control individuals (Tompsett et al. 2012). Thyroid hormones are responsible and necessary for orchestrating metamorphosis (Shi 2000, Das et al. 2010), and exposure to inhibitors of the synthesis of thyroid hormone delay metamorphosis (Degitz et al. 2005). Because a number of genes involved in thyroid hormone signaling and metabolism were already down-regulated at NF stage 53, it is possible that the delays to metamorphosis observed in the 89 d exposure were due, at least in part, to a general down-regulation of the thyroid hormone axis that was detectable weeks before metamorphosis was to occur. In addition, inhibition of synthesis of the thyroid hormones impairs differentiation of the testis and leads to female-biased sex ratios (Goleman et al. 2002). While the sex ratio of animals from the grow-out experiment was not biased toward females, significant effects on differentiation of testes were observed (Tompsett et al. 2012).

There were two biological processes down-regulated in genetic male tadpoles exposed to EE2 that could not be anchored to the phenotypic data collected during the 89 d study: synthesis of cholesterol and steroid and xenobiotic signaling and metabolism. In the 89 d study, endpoints

that are associated with these two pathways were not monitored, so it is not possible to draw conclusions about the possible downstream phenotypic effects of a down-regulation of these processes. Synthesis of cholesterol has been shown to be induced after exposure to potent estrogens (Smith et al. 1978, Sharpe and MacLatchy 2007, Eckman et al. 2009), but most previous studies have monitored cholesterol concentrations, not abundances of transcripts of genes related to cholesterol biosynthesis. In addition to the changes in synthesis of cholesterol, a number of transcripts that are known to be involved in Phase I and II biotransformation reactions (*cyp2c20, cyp2k4-l, gstp1*) were down-regulated by exposure to EE2. CYP2 enzymes have been shown to play a role in metabolism of endogenous hormones, including E2 (Miranda et al. 1989). In addition, inhibitions of members of the CYP2K family of enzymes have been observed in Atlantic salmon (*Salmo salar*) hepatocytes exposed to nonylphenol, a weak estrogen (Thibaut et al. 2002).

3.5.3. Conclusions

Exposure to EE2 during sexual determination and differentiation affected the transcriptome of genetic male *X. laevis* at NF stage 53 in a manner that could be linked with phenotypic data that were collected from a group of *X. laevis* that were exposed to EE2 until after the completion of metamorphosis (89 d). In the current study, analysis of the transcriptome in tadpoles at NF stage 53 was able to identify pathways, processes, and individual genes that might have been associated with the altered phenotypes and delays to metamorphosis that were observed in frogs that were exposed to EE2 for 89 d. Analysis of the transcriptome of exposed individuals by use of *Illumina* sequencing coupled with RNA-Seq was useful for determining the molecular changes that might drive these biologically relevant effects. This technique of analysis of the transcriptome was also helpful in determining *de novo* the pathways and processes that were impacted by exposure to EE2.

CHAPTER 4

Characterization of molecular changes in the liver of African clawed frogs (*Xenopus laevis*) exposed to 17α-ethynylestradiol throughout larval and early post-metamorphic development

4.1. Abstract

The possible contribution of environmental contaminants, including estrogenic compounds, to declines in populations of amphibians is of potential concern. In the current study, transcriptional responses in the liver of the African clawed frog (*Xenopus laevis*) after chronic exposure during the larval period to17α-ethynylestradiol (EE2), the synthetic estrogen used in oral contraceptives, were characterized. Exposure of *X. laevis* to 0.09, 0.84, or 8.81 µg EE2/L had significant effects on abundances of transcripts of genes involved in steroid signaling and metabolism, synthesis of cholesterol, and vitellogenesis. Specifically, among genes involved in steroid signaling and metabolism, abundances of transcripts of farnesoid x-activated receptors α and β were 2.8-8.5-fold less in *X. laevis* exposed to EE2, and abundances of transcripts of the androgen receptor were 5.8-15-fold greater in exposed *X. laevis*. Among genes involved in the synthesis of cholesterol, abundances of transcripts of farnesyl diphosphate synthase were 2.5-4-fold greater in exposed *X. laevis*. Among genes involved in vitellogenesis, abundances of transcripts of estrogen receptor α were 4.5-12-fold greater and abundances of transcripts of vitellogenin A2 were 32-102-fold greater in exposed *X. laevis*. Although there were significant effects on abundances of transcripts associated with exposure to EE2, there were no consistent effects of phenotypic sex on abundances of transcripts. Overall, there were significant effects of exposure to all concentrations of EE2 tested, the least of which was within about 3-fold of estrogen equivalent concentrations routinely measured in the environment.

4.2. Introduction

Some species of amphibian are in decline worldwide (Stuart et al. 2004, Wake 2012) and exposure to chemical contaminants in the aquatic environment has been suggested as a possible threat to the health of amphibian populations (Linder et al. 2003). One class of chemicals that has the potential to harm populations of amphibians is environmental estrogens. Due to their ability to interfere with sexual differentiation, sexual development, and the homeostasis of the endocrine system, estrogens have the potential to impact reproductive endpoints in various species of amphibians (Chang and Witschi 1955, Witschi et al. 1958, Hogan et al. 2008).

Estrogenic substances, such as 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), enter the aquatic environment through sources like the discharge of liquid effluents from wastewater treatment facilities (Ankley et al. 2007). Estrogens are commonly detected in the environment in the ng/L range (reviewed by Corcoran et al. 2010), and there have been demonstrated effects of exposure to these concentrations of estrogens in various aquatic vertebrate species, including fish and frogs (Jobling et al. 1998, Park and Kidd 2005, Kidd et al. 2007, Pettersson and Berg 2007).

Amphibians, including the African clawed frog (*Xenopus laevis*), are relatively sensitive to exposure to estrogens throughout their lifespans. During sexual determination and differentiation (between Nieukwoop-Faber (NF) stages 50 and 54), exposure of *X. laevis* to potent estrogens causes disordered sexual development and even reversal of the male phenotype of genetic males to a female phenotype (Chang and Witschi 1955, Villalpando and Merchant-Larios 1990, Miyata et al. 1999, Lutz et al. 2008, Tompsett et al. 2012). Altered sexual development of genetic males after exposure to estrogens seems to be due to an inhibitory mechanism. Normally, near the time of sexual differentiation, the primordial germ cells of individuals with a male genotype begin to migrate from the cortex to the medulla of the primordial gonad. Treatment with E2 inhibits migration of primordial germ cells in a dose-dependent manner (Hu et al. 2008). However, treatment of *X. laevis* larvae with an aromatase inhibitor to decrease physiological concentrations of estrogens does not masculinize the larvae, indicating that synthesis of endogenous E2 is not necessary for normal differentiation of sexual characteristics in genetic female *X. laevis* (Miyata et al. 1999). In fact, sexually dimorphic expression of the sex hormones is detectable only after sexual differentiation (Bogi et al. 2002). As such, E2 doesn't appear to be necessary for the process of ovarian differentiation in genetic females, but can induce demasculinizing effects on primordial germ cells in genetic males because tadpoles at this stage of development do not normally express great concentrations of endogenous hormones but do have the ability (i.e. receptors) to respond to exposure to hormones.

Exposure to estrogens after the period of sexual differentiation does not cause sex reversal or alter sexual development, but it can impact other biological parameters in *X. laevis*. After NF stage 62, expression of transcripts of the estrogen receptor (ER) and plasma concentrations of E2 become sexually dimorphic and are significantly greater in female individuals than in males (Bogi et al. 2002). This indicates that at some point between NF stages

54 and 62, the ER gene becomes auto-inducible (Tata et al. 1993) and can be up-regulated by the presence of endogenous and exogenous estrogens. Thus, exposure to estrogens after NF stage 62 has the potential to activate genes and pathways that are responsive to estrogens.

Estrogenic compounds, including endogenous hormones, some pharmaceuticals, and certain industrial products, share a similar mode of action, which is the ability to bind to and agonize the ER, thereby inducing expression of estrogen responsive genes (Boelsterli 2003). The induction of these genes presumably leads to the *in vivo* effects observed in vertebrates exposed to estrogenic substances, including reproductive failure and greater production of the yolk-precursor protein vitellogenin in males (reviewed by Sumpter and Johnson 2008). Among the vertebrates, the effects of exposure to estrogens and other endocrine-active compounds tend to be most pronounced in organs that are part of the hypothalamic-pituitary-gonadal-hepatic axis (Watanabe et al. 2009, Villeneuve et al. 2012). Recently, an extensive graphical systems model of the components of this axis in teleost fish was constructed, which included development of tissue specific gene sets affected by endocrine-active compounds in the brain, pituitary, ovary, and liver (Villeneuve et al. 2012). The systems model and enriched gene sets are freely available to members of the scientific community and have great potential for use as tools in ecotoxicological research.

While teleost fish and frogs are not closely related evolutionarily, the typical pathways that are affected by exposure to estrogens are relatively similar among the vertebrates. Results of previous research have indicated that the specific biological pathways affected include vitellogenesis, oocyte maturation and growth, ovulation, and steroid and cholesterol biosynthesis and metabolism (Garcia-Reyero et al. 2009, Watanabe et al. 2009, Villeneuve et al. 2012). These pathways are integral to endocrine homeostasis and reproductive processes, and knowledge about effects of exposure to estrogens on individual components of the pathways can give insight into possible effects at higher levels of biological organization or even at the population or community levels (Garcia-Reyero and Perkins 2011).

The liver is integral in maintaining metabolic homeostasis and is responsible for performing a variety of essential biological processes, including nutrient homeostasis, protein synthesis, and bioactivation, detoxification, and excretion of steroid hormones and xenobiotic compounds (Klaassen 2007). Because of its importance in steroid and xenobiotic metabolism, the liver is a suitable target organ for studying the effects of endocrine active compounds, like

estrogens, on exposed organisms. According to the graphical systems model developed by Villeneuve et al. (2012), the biological processes in the liver most likely to be affected by exposure to endocrine active compounds are steroid signaling and metabolism, cholesterol biosynthesis, and vitellogenesis. Within each of these categories, there are a number of target genes (19-27) that are likely to be affected by exposure to compounds that impact the endocrine system.

The functional gene sets described by Villeneuve et al. (2012) are based upon data from multiple experiments with three different endocrine active compounds, so they might or might not be affected by exposure to estrogens specifically. However, the gene sets offer a suitable starting point for designing experiments to determine the molecular responses of organisms to estrogen exposure. Results of previous research have demonstrated that alterations of the endocrine system can be reliably detected by quantifying abundances of transcripts of target genes by use of quantitative polymerase chain reaction (qPCR) in the amphibian liver (Duarte-Guterman et al. 2010). Thus, the functional gene sets (Villeneuve et al. 2012) were used as a guide in selecting a group of 15 genes to monitor in the amphibian liver using qPCR after chronic exposure to a model estrogen, EE2. These genes included 10 genes involved in steroid signaling and metabolism, 2 genes involved in cholesterol biosynthesis, and 3 genes involved in vitellogenesis (Table 4.1).

The current study utilized a long-term (89 d) exposure of *X. laevis* to determine the effects of exposure to EE2 on sexual development (Tompsett et al. 2012) and expression of genes. *X. laevis* were exposed from before hatch throughout the larval and early post-metamorphic period to nominal doses of 0.1, 1, or 10 µg EE2/L. As such, sexual differentiation and development of genetic male individuals was affected, as has been described previously (Tompsett et al. 2012). In general, a greater proportion of genetic male frogs that were exposed to EE2 developed with altered phenotypes than control genetic males. In the present portion of the study, the objective was to further investigate possible molecular effects of alterations in phenotype by characterization by use of qPCR of molecular responses of a set of 15 transcripts in the liver after chronic exposure to EE2. The concentrations of EE2 chosen for the current study were based upon their ability to elicit effects on phenotype, but the least dose was only 3-fold greater than the range of estrogen equivalents (about 5-30 ng/L) that would be expected in a

natural watershed (reviewed by Kidd et al. 2007) and was 3-fold less than the maximal concentrations of EE2 (about 0.3 µg/L) detected in U.S. streams (Kolpin et al. 2002a, 2002b).

Table 4.1: *Xenopus laevis* primer sets for liver-specific genes expected to be affected by estrogen exposure and a reference gene

Gene Identity	**Abbreviation**	**Accession Number**	**Function**	**Forward primer**	**Reverse primer**
Aryl hydrocarbon receptor 1α	*ahr1a*	AY635782.1	SSM	CATGGTGACTCCCCAGTCTT	GAGCTGCCATGACTGCATTA
Aryl hydrocarbon nuclear translocator 1	*arnt1*	NM_001090153.1	SSM	CTCCTCCCAGAAGCACAAAG	AGCCTCTCGCTGTTCGATAA
Cytochrome P450 1a1	*cyp1a1*	NM_001097072.1	SSM	GATCCGAACCTGTGGAAAGA	CAATGGCTTCACCAACACAC
Estrogen sulfotransferase	*est*	NM_001095815.1	SSM	AATGCCAACGTGACACCATA	ATCAGCTGGATGGGAAGATG
Retinoid X receptor β	*rxrb*	BC108460.1	SSM	TAAGGGCTGGATGGAATGAG	GCCTGCACTGTGTGCACTAT
Pregnane X receptor	*pxr*	NM_001090137.1	SSM	GGTGTCTGCTGGTTGGTTTT	AGTTGTGGGGCTTGATTTTG
Glucocorticoid receptor	*gr*	NM_001088062.1	SSM	TGACATATCTGCCCAACCAA	CCAGGTCCCGTAGTAGGTCA
Farnesoid x-activated receptor α	*fxra*	NM_001088774.1	SSM	TGGAAGCACATCCACAAGAG	TAAGGGATCCCATGCTTCTG
Farnesoid x-activated receptor β	*fxrb*	NM_001090085.1	SSM	TGAAGGCTGCAAAGGTTTCT	CTGACACTTTCTGCGCATGT
Androgen receptor	*ar*	U67129.1	SSM	ATGCTTGTGTGCCAATACCA	TGCAACATGGATGAAGGAAA
7-dehydrocholesterol reductase	*dhcr7*	NM_001086117.1	CB	ATATTCCTGTTGGCGTTTGC	GCGCTTTGCCAGAGTATAGG
Farnesyl diphosphate synthase	*fdps*	NM_001096644.1	CB	CGACGATACGAGGAGGAGAG	GTATCCCAGCATGCCTCACT
Estrogen receptor α	*era*	NM_001089617.1	VTGS	CGACTGGCTCAGCTTCTTCT	AGGGGACAACATTCTTGCAC
High density lipoprotein binding protein	*hdlbp*	NM_001086696.1	VGTS	CACAGGCTATGAGCGAAACA	TTCCGTACAGCTTTGCCTCT
Vitellogenin A2	*vtga2*	NM_001159281.1	VTGS	CTGACTCCTCCCCAAATTCA	CTGTACCAGGTGCTGCAAGA
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	NM_001087098.1	RF	CAGAGGTGCAGGTCAGAACA	GGAAAGCCATTCCGGTTATT

SSM=steroid signaling and metabolism; VTGS=vitellogenesis; CB=cholesterol biosynthesis; RF=reference

4.3. Materials and methods

4.3.1. *Xenopus laevis*

Prior to commencement of research, approval for the use of animals and all experimental procedures was obtained from the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan (Animal Use Protocol #20090066). Sexually-mature, adult *X. laevis* were purchased from Boreal Laboratories (St. Catharine's, ON, Canada) and acclimated to laboratory conditions (18±2 °C water temperature; 12:12 light-dark cycle; fed Nasco frog brittle [medium nuggets] (Salida, CA, USA) *ad libitum* daily) for one month. After acclimation, male and female pairs of *X. laevis* were spawned and eggs were collected as has been described previously (Tompsett et al. 2012).

4.3.2. 17α-ethynylestradiol exposure

The exposure was conducted as has been detailed previously (Tompsett et al. 2012). Briefly, each treatment group consisted of triplicate tanks with 15 individuals per tank. The appropriate nominal concentration of EE2 was added to each tank in an ethanol carrier, and the final concentration of ethanol in treatment tanks was 0.0025%. A 50% static water renewal was performed daily and basic water quality variables were monitored daily (temperature, DO, pH, conductivity) or weekly (nitrate nitrogen, nitrite nitrogen, ammonia nitrogen). Tadpoles and frogs were fed *ad libitum* daily with Nasco frog brittle products.

4.3.3. Analysis of 17α-ethynylestradiol concentrations

To validate that expected nominal values were approximated, concentrations of EE2 in exposure water were monitored periodically during the exposure via high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). EE2 was quantified by use of methods that have been described elsewhere (Chang et al. 2010). Briefly, samples of whole

water were spiked with a deuterated internal EE2 standard (C/D/N Isotopes, Inc., Pointe-Claire, QC, Canada), extracted two times with hexane and then concentrated under nitrogen. Dried organic extracts were derivatized by use of dansyl chloride, re-extracted with hexane, dried under nitrogen, and then reconstituted in acetonitrile. Analytical detection was conducted by use of an Agilent 1200 series HPLC system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole MS/MS system (PE Sciex, Concord, ON, Canada). Both the LC and the mass spectrometer were controlled by AB Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA, USA).

4.3.4. Termination of exposure and determination of phenotypic and genetic sex

X. laevis were exposed to EE2 for 89 d. At termination of the exposure, euthanasia was performed by an overdose of MS-222 (Sigma). The liver was removed from each froglet, flash frozen in liquid nitrogen, and stored at -80 °C until analysis of abundances of transcripts was performed. The gonadal phenotype of each individual was determined as has been reported previously (Tompsett et al. 2012). Briefly, gonads were examined for the presence of male and female tissues, and it was assessed whether those tissues were of normal morphology or not (Ankley et al. 2006). Individuals were classified as male, female, mixed sex, or abnormal male (Hecker et al. 2006). Abnormal males were characterized by a lack of spermatocysts and/or abnormal shape of the testis.

In *X. laevis*, the *DM-W* gene can be used as a marker of female genetic sex, and its absence is a marker of male genetic sex. A simple PCR assay has been developed to determine genetic sex in *X. laevis* individuals (Yoshimoto et al. 2008). The assay uses a genomic DNA sample and, thus, can be performed on any tissue, including pieces of tadpole tails and toe clips. The genotype of each froglet was determined using this assay with a tissue sample taken from the leg as has been previously described (Tompsett et al. 2012).

For quantification of abundances of transcripts, each individual frog was assigned to one of the four following groups based on its genetic and phenotypic sex: genetic and phenotypic female, genetic and phenotypic male, genetic male and abnormal phenotypic male, or genetic male and mixed sex phenotype. In addition to this classification by sex category, abundances of

transcripts were also evaluated on the basis of treatment group. In total, 14 combinations of sex and treatment group were analyzed.

4.3.5. RNA Isolation, complimentary DNA synthesis, design of primers, and quantitative polymerase chain reaction

Total RNA was isolated from livers of *X. laevis* by use of an RNeasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. RNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Integrity of RNA was checked on a 1% denaturing formaldehyde-agarose gel with ethidium bromide and visualized under ultraviolet light on a VersaDoc 4000MP imaging system (BioRad, Mississauga, ON, Canada). Samples of purified RNA were stored at -80 °C until analysis. First-strand complimentary DNA (cDNA) was synthesized from 1 µg of each RNA sample using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. The cDNA samples were stored at -80 °C until further analysis.

Primers for use in qPCR were designed for genes of interest (Table 4.1) using published *X. laevis* mRNA sequences (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) and Primer3 software (Rozen and Skaletsky 2000). Primers were designed to amplify PCR products between 100 and 150 base pairs in length using standard parameters, and forward and reverse DNA oligonucleotides (oligos) were purchased (Invitrogen, Burlington, ON, Canada).

For all primer sets, efficiency curves were constructed to determine their suitability for qPCR. Briefly, for each primer set, qPCR was performed on a standard curve consisting of cDNA dilutions (undiluted, 1:5, 1:25, 1:125, 1:625, and 1:3125) of a pool of cDNA from 5 random livers of *X. laevis* using a Quantitect SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol. Further qPCR experiments were performed for those primers with standard curves with a coefficient of determination (R^2) of at least 0.99 and efficiencies of 1.9-2.1, where efficiency=$10^{(-1/\text{slope of standard curve})}$.

Abundances of transcripts of 15 target genes (Table 4.1) were evaluated for 48 individual samples of cDNA from 14 combination sex/treatment categories using qPCR. The sample size

for each treatment and sex category varied according to the number of samples available in each classification. For the control and solvent control treatments, 2 genetic and phenotypic females and 2 genetic and phenotypic males from each triplicate tank were analyzed, for a total of 6 individuals per category. For the individuals exposed to different concentrations of EE2, at least 1-2 genetic and phenotypic females and 1-2 genetic and abnormal males from each triplicate tank were analyzed, for a total of 4 individuals per category. For the rest of the treatment/phenotype combinations, there were only 3 individuals per category available, and all were used. These categories included genetic and phenotypic males exposed to 0.09 µg EE2/L and genetic males with a mixed sex phenotype from all EE2 treatment concentrations.

To perform qPCR, cDNA from each individual liver was diluted 1:5, and a QuantiTect SYBR Green PCR Kit (Qiagen) was used according to the manufacturer's protocol. Briefly, a separate 50 µL PCR reaction consisting of SYBR Green master mix, gene-specific primers, nuclease free water, and cDNA was prepared for each cDNA sample and primer pair. Then, duplicate 20 µL reactions were transferred to a 96-well PCR plate. The qPCR was performed in an ABI 7300 Real-Time PCR System (Applied Biosystems, Burlington, ON, Canada). The PCR reaction mixture was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was as follows: denature for 15 sec at 95 °C and extension for 1 min at 60 °C for a total of 40 PCR cycles. The qPCR cycle was followed by a dissociation step to validate that all cDNA samples amplified only a single product. For each target gene, abundance of transcripts was quantified according to the Mean Normalized Expression (MNE) method of Simon (2003) with glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) as a reference gene.

4.3.6. Statistics

Statistical tests were performed using IBM SPSS 19 software (IBM, Armonk, NY). Treatment means are expressed as mean ± S.E. throughout. Statistical significance was defined as $p \leq 0.05$. Statistical analyses of genotype and phenotype data were performed as previously described (Tompsett et al. 2012). Data for abundances of transcripts of genes of interest were first assessed for normality and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively, and evaluated for statistical equivalency between control and solvent control

treatments and pooled where appropriate. Data for all transcripts deviated from normality and/or homogeneity of variances, so they were analyzed with nonparametric Kruskal-Wallis tests to determine differences among treatments in abundances of transcripts. Where the p-value of the Kruskal-Wallis test was significant, post-hoc testing was performed using Mann-Whitney U tests to compare each treatment group to the control female group.

4.4. Results

4.4.1. Water quality and validation of 17α-ethynylestradiol concentrations

Water quality variables over the course of the experiment were all within an acceptable range for culture of *X. laevis* and have been reported previously (Tompsett et al. 2012). Validated concentrations of EE2 in treatment tanks have also been reported previously (Tompsett et al. 2012). Briefly, actual concentrations in EE2 treatment tanks were 0.09 ± 0.005, 0.84 ± 0.06, and 8.81 ± 1.25 µg EE2/L immediately following water changes. After 24 hr, mean concentrations of EE2 were 20%, 43%, and 28% less than after water change in the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively. Since EE2 concentrations after 24 hr were not monitored as often as concentrations immediately following water change, validated concentrations of EE2 after water change are used hereafter to designate treatment groups.

4.4.2. Genetic and phenotypic sex ratios

All females from all treatments were classified as phenotypic females possessing histologically normal ovarian tissue, although some individuals did exhibit abnormal expression of vitellogenin protein that has been discussed previously (Tompsett et al. 2012). Among genetic males, there were significant differences between treatments in the proportion of male, abnormal male, mixed sex, and female phenotypes (Fisher's Exact test, $p<0.0001$; Figure 2.6). In the control and solvent control treatments, 0% and 5% of genetic males had altered phenotypes, while 83%, 93%, and 92% of genetic males in the 0.09, 0.84, and 8.81 µg EE2/L treatments had

altered phenotypes, respectively. In general, EE2 treatments had a lesser proportion of normal males and a greater proportion of abnormal males. In addition, the 8.84 µg EE2/L treatment had significantly greater proportions of both mixed sex and sex-reversed phenotypic females.

4.4.3. Abundance of transcripts of target genes

4.4.3.1. Genes involved in steroid signaling and metabolism

There were 10 genes involved in steroid signaling and metabolism monitored in the current study. Of these genes, the abundances of transcripts of farnesoid X-activated receptor α (*fxra*), farnesoid X-activated receptor β (*fxrb*), and androgen receptor (*ar*) were significantly altered in *X. laevis* after exposure to EE2 (Kruskal-Wallis, p= 0.002, 0.006 and 0.014 for *fxra, fxrb,* and *ar*, respectively). For *fxra*, fold-changes in abundances of transcripts that were significantly less than control females, ranging from -8.4-fold to -2.8-fold, were present in abnormal male, mixed sex, and female individuals exposed to 0.84 µg EE2/L and in abnormal male and female individuals exposed to 8.81 µg EE2/L (Table 4.2). For *fxrb*, fold-changes in abundances of transcripts that were significantly less than control females, ranging from -8.5-fold to -3.4-fold, were present in abnormal male and female individuals exposed to 0.84 µg EE2/L and female individuals exposed to 8.81 µg EE2/L (Table 4.2). For *ar*, fold-changes in abundances of transcripts that were significantly greater than control females, ranging from +5.8-fold to +15-fold, were present in all of the EE2 treated groups, except mixed sex individuals exposed to 8.81 µg EE2/L (Table 4.2; Figure 4.1). There was no change in the abundance of transcripts of the aryl hydrocarbon receptor 1 α (*ahr1a*), cytochrome P450 1a1 (*cyp1a1*), aryl hydrocarbon nuclear translocator 1 (*arnt1*), estrogen sulfotransferase (*est*), retinoid X receptor β (*rxrb*), glucocorticoid receptor (*gr*), or pregnane X receptor (*pxr*).

4.4.3.2. Genes involved in cholesterol biosynthesis

Abundances of transcripts of two genes involved in synthesis of cholesterol in liver, 7-dehydrocholesterol reductase (*dhcr7*) and farnesyl diphosphate synthase (*fdps*), were evaluated. There was no change in the abundance of transcripts of *dhcr7*. There were significant differences among treatments in the abundances of transcripts of *fdps* (Kruskal-Wallis, p=0.013). The abundances of transcripts of *fdps* were significantly greater than control females, ranging from +2.6-fold to +4.0-fold, in males, abnormal males, and females exposed to 0.09 µg EE2/L and in abnormal males and females exposed to 0.84 µg EE2/L (Table 4.2).

4.4.3.3. Genes involved in vitellogenesis

Abundances of transcripts of three genes that are involved in production of vitellogenin were examined. These included estrogen receptor α (*era*), high density lipoprotein binding protein (*hdlbp*), and vitellogenin A2 (*vtga2*). Abundances of transcripts of *era* and *vtga2* were significantly altered among treatments (Kruskal-Wallis, p=0.003 and 0.002 for *era* and *vtga2*, respectively), but abundances of *hdlbp* were not altered by exposure to EE2. For *era*, the abundances of transcripts were significantly greater than control females, ranging from +4.5-fold to +12-fold, in all EE2 treatment groups, except mixed sex individuals exposed to both 0.84 and 8.81 µg EE2/L (Table 4.2; Figure 4.2). For *vtga2*, the abundances of transcripts were significantly greater than control females, ranging from +32-fold to +102-fold, in all EE2 treatment groups, except mixed sex individuals exposed to 8.81 µg EE2/L (Table 4.2; Figure 4.3).

Table 4.2: Summary of fold-changes in relative abundances of transcripts in liver of *Xenopus laevis* after exposure to 17α-ethynylestradiol during development.

17α-ethynylestradiol concentration		0.09 µg/L				0.84 µg/L			8.81 µg/L		
Phenotype		M	AM	MS	F	AM	MS	F	AM	MS	F
Transcript	**Biological function**	**Fold-change[a] (±S.E.)**									
fxra	SSM	-	-	-	-	-3.4 (±0.1)	-8.4 (±0.1)	-3.8 (±0.1)	-2.8 (±0.2)	-	-8.1 (±0.1)
fxrb	SSM	-	-	-	-	-3.9 (±0.1)	-	-3.4 (±0.1)	-	-	-8.5 (±0.1)
ar	SSM	+11 (±2.3)	+6.7 (±3.2)	+7.5 (±0.7)	+6.9 (±1.5)	+6.9 (±2.3)	+8.5 (±3.2)	+7.4 (±1.1)	+15 (±6.5)	-	+5.8 (±2.1)
era	VTGS	+12 (±2.1)	+7.2 (±3.5)	+8.8 (±3.9)	+7.3 (±1.5)	+6.8 (±1.7)	-	+4.5 (±0.9)	+6.6 (±3.9)	-	-
vtga2	VTGS	+102 (±22)	+86 (±51)	+71 (±36)	+84 (±16)	+52 (±15)	+87 (±30)	+38 (±11)	+61 (±31)	-	+32 (±14)
fdps	CB	+2.9 (±0.8)	+2.8 (±0.4)	-	+4.0 (±0.5)	+2.7 (±0.8)	-	+2.6 (±0.3)	-	-	-

M=male; AM=abnormal male; MS=mixed sex; F=female; SSM=steroid signaling and metabolism; VTGS=vitellogenesis; CB=cholesterol biosynthesis

[a] Data are presented for those transcripts that were significantly altered (Kruskal-Wallis, $p \leq 0.05$; post-hoc Mann-Whitney U, $p \leq 0.05$; *n*=3-6 frogs in each category) in individuals exposed to EE2 compared to control female individuals. A dash indicates that the fold-change was not significant.

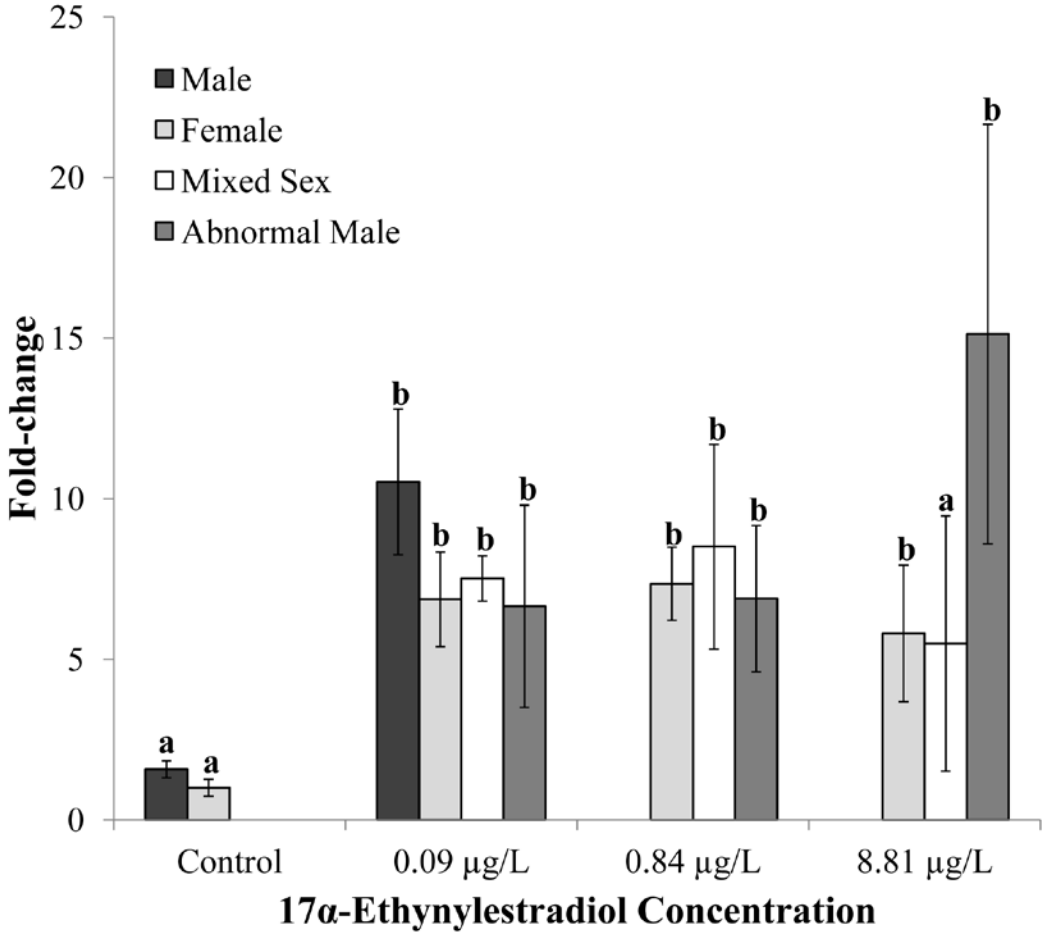

Figure 4.1: Fold-changes in abundances of transcripts of androgen receptor (*ar*) in the livers from *Xenopus laevis* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of transcripts of *ar* among treatments after exposure. Significant differences from control treatments are denoted by different letters (Kruskal-Wallis, p=0.014; *n*=3-6 frogs in each category).

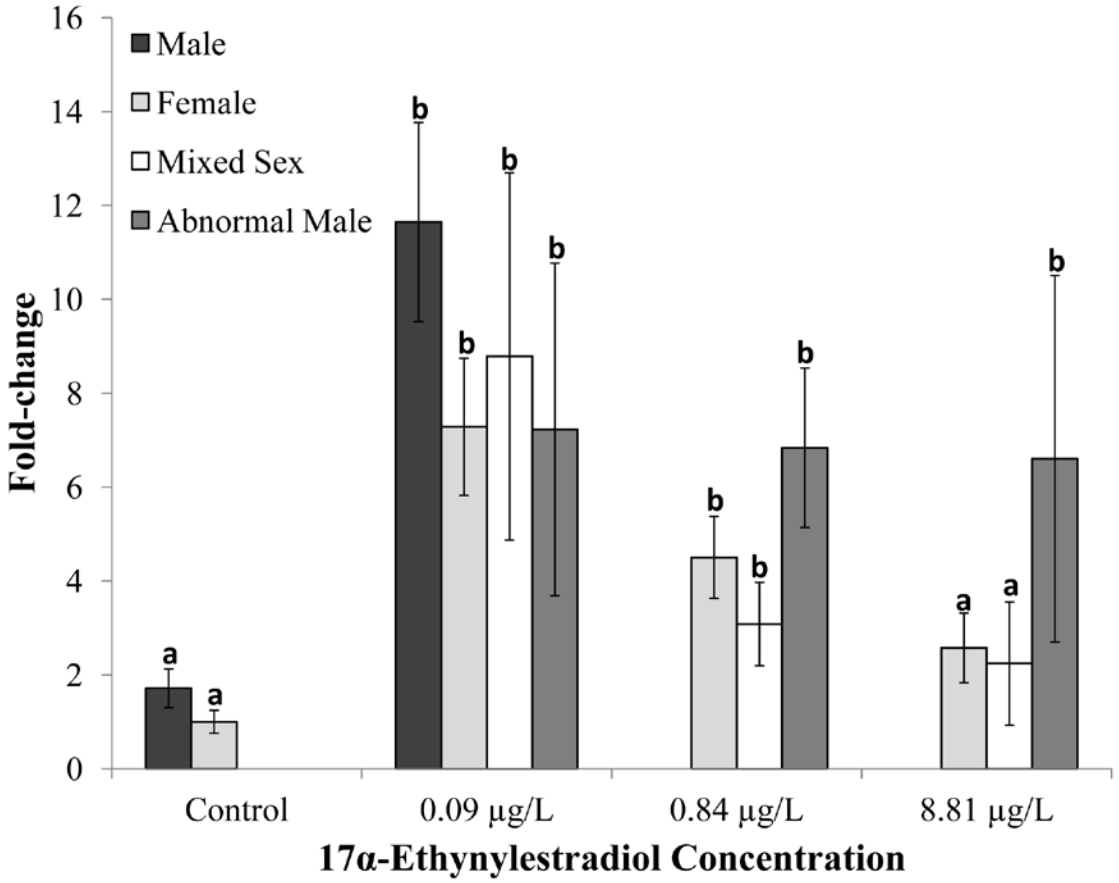

Figure 4.2: Fold-changes in abundances of transcripts of estrogen receptor α (*era*) in the livers from *Xenopus laevis* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of transcripts of *era* among treatments after exposure. Significant differences from control treatments are denoted by different letters (Kruskal-Wallis, p=0.003; *n*=3-6 frogs in each category).

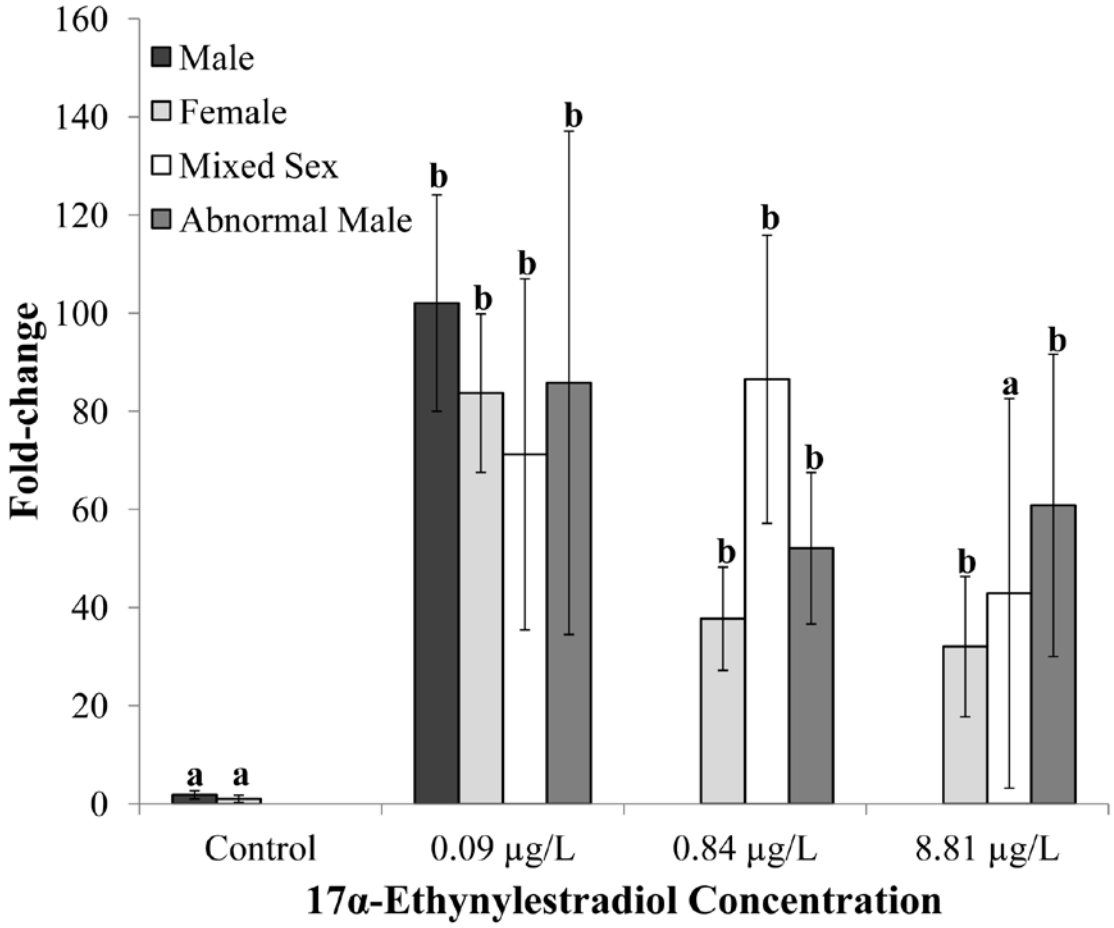

Figure 4.3: Fold-changes in abundances of transcripts of vitellogenin A2 (*vtga2*) in the livers from *Xenopus laevis* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of transcripts of *vtga2* among treatments after exposure. Significant differences from control treatments are denoted by different letters (Kruskal-Wallis, p=0.002; *n*=3-6 frogs in each category).

4.5. Discussion

In the current study, long-term exposure of *X. laevis* to EE2 at concentrations within the realm of environmental concern significantly altered the abundance of transcripts of genes that are involved in metabolism of steroids, synthesis of cholesterol, and vitellogenesis in liver. These changes were detectable after 89 d of constant exposure, which suggested that alterations in abundances of certain transcripts might be a suitable endpoint in subchronic and chronic studies of the effects of environmental estrogens on amphibians. Previous experiments with *Silurana (Xenopus) tropicalis* exposed chronically to the model aromatase inhibitor fadrozole or the 5-reducatase inhibitor finasteride from NF stages 12-60 found that the abundances of transcripts of genes involved in steroid metabolism and vitellogenesis were altered in the liver (Duarte-Guterman et al. 2010). Therefore, the results of that study and of the current study indicate that abundances of transcripts of genes involved in endocrine functions can be suitable indicators of chronic exposure to endocrine-active chemicals.

For all 15 genes examined in the current study (Table 4.1), there was no consistent effect of phenotypic sex on the abundances of gene transcripts. However, exposure to EE2 during the larval period did significantly affect phenotypic sex ratios and normal sexual development of *X. laevis*. In general, exposure to all concentrations of EE2 tested in the current study altered sexual differentiation and development of a significant proportion of individuals with a male genotype, which led to greater proportions of genetic males with abnormal male, mixed sex, and female phenotypes after exposure to EE2 (Tompsett et al. 2012). At the concentrations of EE2 tested, 83-93% of genetic males had an altered phenotype. However, abundances of transcripts of target genes in individuals with altered phenotypes were not consistently different from those of normal individuals that were also exposed to EE2.

Altered gene expression has been linked to altered phenotype in the amphibian *S. tropicalis*. The abundances of transcripts of a number of 5-reductases were changed in the liver of chemically-induced mixed sex (phenotypic males with testicular oocytes) *S. tropicalis* after exposure to finasteride (Duarte-Guterman et al. 2010). There are a number of reasons that *S. tropicalis* individuals with a mixed sex phenotype might have been more responsive than the *X. laevis* with a mixed sex phenotype in the current study. While treatment with both EE2 and finasteride result in feminization or desmasculinization, the two chemicals do not share a similar

mode of action and do not affect expression of the same genes. EE2 is an ER agonist, while finasteride directly inhibits the reductase enzymes responsible for converting testosterone into dihydrotestosterone. In addition, while individuals from both studies were classified as mixed sex, testicular oocytes were rare in the current study but were present in all mixed sex individuals in the study by Duarte-Guterman et al. (2010). The differences between the two studies might also be explained by biological differences between *X. laevis*, which takes 1-2 years to reach sexual maturity, and *S. tropicalis*, which takes only 4-6 months to reach maturity (Hirsch et al. 2002). It is possible that all individuals from the current study were too immature to display sexually dimorphic differential expression of genes involved in endocrine processes after chronic exposure, no matter the chemical stressor they were exposed to.

While there was no consistent impact of phenotypic sex on the abundances of transcripts in the livers, there were significant differences in the abundances of transcripts between individuals exposed to EE2 and control individuals for 6 of the 15 genes assessed in the current study. The remainder of discussion herein focuses on differences in abundances of transcripts between EE2 treated individuals and control individuals in the three functional groups of genes evaluated: genes involved in steroid signaling and metabolism, genes involved in synthesis of cholesterol, and genes involved in vitellogenesis.

4.5.1. Genes involved in steroid signaling and metabolism

The abundances of transcripts of *fxra* and *fxrb* were significantly altered in the liver of *X. laevis* after chronic exposure to EE2. The farnesoid X receptors (FXRs) are involved in bile acid transport and metabolism in vertebrates, and their natural ligands are bile acids, which are end metabolites of cholesterol (Reschly et al. 2008). Non-mammalian vertebrates, such as fish, secrete metabolites of steroids and xenobiotics into bile acids produced in the liver during detoxification (Gibson et al. 2005). Similar mechanisms are common to mammals, which also transcriptionally regulate some nuclear receptors, including RXRs, PXRs, and FXRs, as a hepatoprotective mechanism (Zollner and Trauner 2009, Henriquez-Hernandez et al. 2007). In addition, lesser abundances of transcripts of *fxr* in the human liver have been linked to the presence of primary liver tumors (Martinez-Beccerra et al. 2012). In the current study,

abundances of transcripts of both *fxra* and *fxrb* were significantly less than abundances in control groups in some phenotype groups exposed to both 0.84 and 8.81 µg EE2/L. The lesser abundances of transcripts of these receptors might indicate that the hepatoprotective functions served by these receptors were also adversely affected.

Abundances of transcripts of *ar* were significantly greater than control abundances in all groups of *X. laevis* exposed to EE2, except for mixed sex individuals exposed to 8.81 µg EE2/L. The balance of estrogens and androgens is an important part of endocrine homeostasis (Rivas et al. 2002). Although the two classes of hormones have differing functions in reproduction, the formation of endogenous E2 is dependent upon aromatization of testosterone (T) by the aromatase enzyme (CYP19). Although both E2 and T are ligands for the ER and AR, respectively, there is evidence that the ER and AR are not exclusively agonized by E2 and T, and there is the potential for crosstalk between the two pathways (Eick and Thornton 2011). For example, in the human prostate, 3β-adiol, a metabolite of dihydrotestosterone, is a known agonist of ERβ (Muthusamy et al. 2011). In the current study, exposure to EE2, an agonist of the ER, resulted in greater abundance of transcripts of the *ar*. While this is not a response that is typically associated with exposure to estrogens, the chronic nature of the study and the potential for interactions between the AR- and ER-mediated pathways could explain the effect. The endocrine system is regulated by feedback loops, and it is possible that the greater abundance of transcripts of *ar* is an indication that these frogs were attempting to maintain homeostatic balance between *ar* and *er* signaling pathways via greater transcription of *ar*.

Since in the current study *X. laevis* were chronically exposed to EE2 for 89 d, it is somewhat surprising that genes involved in metabolizing and detoxifying steroids, especially *est*, were not up-regulated to a greater extent. The enzyme coded for by the *EST* gene is a sulfotransferase that preferentially metabolizes endogenous estrogens and facilitates their elimination from circulation (Kauffman 2004). Studies with human estrogen-specific enzymes have shown that EE2 is a suitable substrate for sulfotranferases, and that EE2 can be sulfated by some isoforms at greater rates than E2 (Yasuda et al. 2005). Although all *X. laevis* from the current study were exposed to relatively great concentrations of EE2, the abundance of transcripts of *est* were not greater in individuals from any EE2 treatment groups. While it is unknown why transcription of *EST* was not affected by exposure to EE2, it could be that there was a transient increase in transcription that was not captured due to the chronic nature of the

study. It is also possible that there were interactions between *EST* and other genes that suppressed transcription (Kodama et al. 2011) or that activity of the enzyme coded for by the EST gene would have been a better measure of the potential for metabolism of estrogens.

4.5.2. Genes involved in cholesterol biosynthesis

The FDPS enzyme catalyzes the formation of farnesyl diphosphate, a precursor of sterols, carotenoids, and ubiquinones, which is an intermediate product in formation of cholesterol. In the current study, abundances of transcripts of *fdps* were significantly greater in some groups of individuals exposed to 0.09 and 0.84 µg EE2/L. However, abundances of transcripts of *dhcr7*, which codes for the enzyme that catalyzes the final step of synthesis of cholesterol, were not affected by exposure to EE2. In other non-mammalian vertebrates, exposure to estrogens affects synthesis of cholesterol. In female fathead minnows (*Pimephales promelas*), exposure to EE2 increases total cholesterol in the liver after 4-8 d of exposure (Ekman et al. 2009). Concentrations of cholesterol were also greater in livers of goldfish (*Carassius auratus*) after 5 months of exposure to E2 (Sharpe and MacLatchy 2007). In addition, *X. laevis* livers produce greater amounts of fatty acids and cholesterol after exposure to estrogen (Smith et al. 1978). In the current study, concentrations of cholesterol were not monitored, so it is unclear whether synthesis of cholesterol was ultimately affected by exposure to EE2, especially since the transcripts that were monitored displayed differential responses to EE2.

4.5.3. Genes involved in vitellogenesis

Transcripts of three genes involved in vitellogenesis, *era, vtga2,* and *hdlbp*, were examined in the current study. In general, abundances of transcripts of *era* and *vtga2* were significantly greater in individuals exposed to EE2, while abundances of transcripts of *hdlbp* were not changed by exposure to EE2. Functional relationships between the genes affected by exposure to EE2, and the relationship of these genes with estrogen, readily explain the greater abundances of transcripts measured in the current study. Vitellogenin (VTG) is a yolk-precursor

protein that is normally produced by female organisms that are preparing to generate eggs to spawn. The gene coding for VTG is present in both genetic male and female organisms, but, under normal circumstances, males and juveniles do not express great quantities of it. However, expression of VTG is rapidly induced in response to exposure to estrogens, even in genetic males, via binding of an activated ER complex to an estrogen response element in the promoter region of the gene (reviewed by Rotchell and Ostrander 2003). Thus, in the context of the current study, abundances of transcripts of *era* and *vtga2* were greater due to a positive-feedback loop where exposure to EE2 increases expression of both genes, even in sexually immature or male individuals that might not normally express them.

Greater abundances of transcripts of *vtga2* measured in the current study complement the greater amounts of VTG protein that was described in these same *X. laevis* (Tompsett et al. 2012). Histological analysis of the kidney-gonad complexes of these *X. laevis* revealed proteinaceous material in and around the gonads and kidneys that was identified, using immunohistochemistry, as VTG (Figure 2.7). While there has been considerable debate over the biological relevance of up-regulated VTG expression, it was hypothesized that utilization of energy resources to produce VTG possibly contributed to the significant delays to metamorphosis observed after exposure of *X. laevis* to EE2 (Tompsett et al. 2012). Since synthesis of proteins is an energetically expensive process that can account for nearly 80% of cellular oxygen consumption in isolated hepatocytes of rainbow trout (Pannevis and Houlihan 1992), it is feasible that intensive production of VTG exhausted energy stores needed for completion of metamorphosis. In addition, even though these frogs showed evidence of VTG protein deposits in the kidney-gonad complex, abundances of *vtga2* transcripts were still elevated, suggesting that vitellogenesis was still occurring.

4.5.4. Conclusions

Analysis of abundances of transcripts of genes related to steroid signaling, synthesis of cholesterol, and vitellogenesis in the livers of sexually immature *X. laevis* by use of qPCR indicated that alerations in the abundances of some of these genes could be measured even after 89 d of exposure to EE2. These changes illustrate that qPCR can be a useful technique even in

scenarios where exposure is chronic. For studies utilizing chemicals like EE2, this is especially useful since environmentally realistic exposures are likely to be chronic due to the constant input of estrogens into the aquatic environment via sources such as effluents from wastewater treatment plants and runoff from agricultural operations. In addition, the observed changes in abundances of transcripts were driven by exposure to EE2 and not by phenotypic sex, even in control individuals. This suggests that sexually immature *X. laevis* might be sensitive indicators of exposure to estrogenic compounds like EE2, since they are not yet mature enough to express transcripts related to endocrine function in a sexually dimorphic manner.

CHAPTER 5

Effects of exposure to 17α-ethynylestradiol during development on growth, sexual differentiation, and hepatic gene expression in larval wood frogs (*Rana sylvatica*)

5.1. Abstract

Populations of amphibians are in decline in locations around the world, and the possible contribution of environmental contaminants, including estrogenic compounds, to these declines is of potential concern. In the current study, the responses of the wood frog (*Rana sylvatica*) to exposure to17α-ethynylestradiol (EE2), the synthetic estrogen used in oral contraceptives, during the larval period were characterized. Exposure of *R. sylvatica* to 1.08, 9.55, or 80.9 µg EE2/L had no effects on survival, growth, or metamorphic endpoints monitored in the current study. However, there were significant effects of exposure to EE2 on phenotypic sex ratios. In general, lesser proportions of *R. sylvatica* developed as phenotypic males and greater proportions developed as phenotypic females or with mixed sex phenotypes at all concentrations of EE2 tested. Utilizing the data collected in the current study, the EC_{50} for complete feminization of *R. sylvatica* was determined to be 7.7 µg EE2/L, and the EC_{50} for partial feminization was determined to be 2.3 µg EE2/L. In addition, after chronic exposure, abundances of transcripts of vitellogenin A2, high density lipoprotein binding protein, and 7-dehydrocholesterol reductase were 1.8- to 280-fold greater in livers from *R. sylvatica* exposed to EE2 compared to control *R. sylvatica*. Overall, there were significant effects of exposure to all concentrations of EE2 tested, the least of which was within about 2-fold of estrogen equivalent concentrations previously measured in the environment.

5.2. Introduction

Declining populations of amphibians are of concern worldwide (Stuart et al. 2004, Wake 2012). Several factors have been considered as possible contributing causes to these declines, and these factors include exposure to chemical contaminants in the aquatic environment (Linder et al. 2003). One class of contaminants that is of concern to amphibians is those that can modulate endocrine function. Some of these chemicals can be released by human activities and might potentially affect endocrine systems of aquatic vertebrates. Among the endocrine active chemicals, environmental estrogens have been hypothesized to be one group of chemicals that might affect populations of amphibians, due to their ability to feminize and/or demasculinize

development and homeostasis of the endocrine system, thereby potentially affecting reproductive fitness (Witschi 1951, Chang and Witschi 1955, Hogan et al. 2008). Estrogenic substances, such as 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), can enter the aquatic environment through sources like the discharge of liquid effluents from wastewater treatment facilities (Ankley et al. 2007) and runoff that contains animal manure (Hanselman et al. 2003), which has the potential to result in the exposure of aquatic wildlife, including fish and amphibians.

The wood frog (*Rana sylvatica*) is native to areas of North America. It ranges further north than any other species of frog in North America and is the only species of frog found north of the Arctic Circle. *R. sylvatica* are considered common throughout much of their range, but they are not generally used in toxicological studies that focus on the effects of endocrine active chemicals. Other native species of North American frogs in the family Ranidae, like green frogs (*Rana clamitans*) (Park and Kidd 2005, McDaniel et al. 2008), bullfrogs (*Rana catesbieana*) (Veldhoen and Helbing 2001, Gunderson et al. 2011), and Northern or Southern leopard frogs (*Rana pipiens* or *Rana sphenocephala*) (Tsai et al. 2005, Hogan et al. 2008, Storrs and Semlitsch 2008, Langlois et al. 2010), are more commonly used. However, considering their extensive range in comparison with other North American species, additional toxicological data pertaining to *R. sylvatica* would be relevant to assessments of risks posed by estrogenic chemicals in a variety of ecosystems. While *R. sylvatica* are not a typical non-model species, these frogs are amenable for use in laboratory (Mackenzie et al. 2003, Howe et al. 2004, Hogan et al. 2006) and field studies (Heggstrom 2009, Hersikorn and Smits 2011).

R. sylvatica are sensitive to exposure to estrogens around the time of sexual differentiation. Exposure to concentrations of potent estrogens in the 10-100 µg/L range at this time results in reversal of the phenotypic sex of genetic males, meaning they develop as phenotypic females (Witschi 1951). While mechanisms responsible for reversing the phenotypic sexual characteristics of males to those of females in *R. sylvatica* are not clear, previous research with the African clawed frog (*Xenopus laevis*) indicates that changes of genetic males to phenotypic females is due to the failure of primordial germ cells to migrate after exposure to estrogen (Hu et al. 2008). Thus, the reversal of phenotypic sex is due to demasculinizing effects of estrogen, which would not normally be present in great quantities in genetic males. Although *R. sylvatica* has an XY system of sex determination, while *X. laevis* has a ZW system of sex

determination, it is possible that a similar mechanism is responsible for reversal of phenotypic sex in both species.

Exposure to estrogens after sexual differentiation has been completed cannot cause sex reversal, but it can affect other biological parameters in amphibians. During larval development, the estrogen receptor (ER) gene becomes auto-inducible, which means it is able to be up-regulated by the presence of endogenous or exogenous estrogens (Tata et al. 1993). Once the ER becomes auto-inducible, exposure to estrogens activates typical estrogen-responsive pathways that are commonly used in studies of estrogenic compounds in a variety of vertebrate species (Garcia-Reyero et al. 2009, Watanabe et al. 2009, Villeneuve et al. 2012). Estrogenic compounds, including endogenous hormones, pharmaceuticals, and synthetic chemicals, share a similar mode of action, which is the ability to bind to and agonize the estrogen receptor, thereby inducing expression of estrogen responsive genes (Boelsterli 2003). Induction of these genes presumably leads to *in vivo* effects observed in aquatic vertebrates exposed to estrogenic substances, including reproductive failure, formation of mixed sex gonads, and greater production of the yolk-precursor protein vitellogenin (reviewed by Sumpter and Johnson 2008). In vertebrates, effects on estrogen-responsive genes are most pronounced in organs that are part of the hypothalamic-pituitary-gonad-liver axis (Villeneuve et al. 2012). Previous studies have demonstrated that alterations of the endocrine system can be reliably detected by monitoring expression of genes in amphibian liver (Duarte-Guterman et al. 2010, Chapter 4 of this thesis). Specific genes in the liver affected by exposure to estrogens can be classified into three functional groups: genes involved in steroid metabolism and signaling, genes involved in vitellogenesis, and genes involved in synthesis of cholesterol (Villeneuve et al. 2012).

EE2 is the synthetic estrogen used in human oral contraceptives, and it is structurally and functionally similar to E2 *in vivo*. The initial work with *R. sylvatica* exposed to EE2 during sexual differentiation indicated that concentrations of 1 µg EE2/L had no effect on sex ratios, 10 µg EE2/L partially feminized/demasculinized male larvae, and concentrations of 100 µg EE2/L completely feminized/demasculinized male larvae (Witschi 1951). However, a later study indicated that exposure of *R. sylvatica* tadpoles to 1 or 10 µg EE2/L had no effects on sexual differentiation, although that study suffered from some design flaws, including no replication of EE2 treatments (Mackenzie et al. 2003). Thus, although the threshold for feminization or demasculinization of the *R. sylvatica* by EE2 is unclear, exposure to 100 µg EE2/L significantly

affected sexual differentiation of genetic males. This concentration elicits effects on phenotype, but it is ~6–fold less than the LC_{50} (~568 µg EE2/L) and ~2–fold less than the threshold for biological effects on growth (~225 µg EE2/L) for *R. sylvatica* (Hogan et al. 2006).

The current study was designed to utilize the model estrogen EE2 to cause disordered sexual development in *R. sylvatica*, and to further characterize concentrations of EE2 that cause feminization/demasculinization in this species. In addition, to determine the effects of exposure on other biological processes, abundances of transcripts of genes involved in steroid metabolism and signaling, synthesis of cholesterol, and vitellogenesis were evaluated in livers from affected individuals after long-term (up to 100 d) exposure to EE2. Individuals were exposed to EE2 throughout larval development and euthanized upon reaching metamorphic climax. Based on previously published results, nominal concentrations of EE2 chosen for the current study, 1, 10, and 100 µg EE2/L, were expected to cause few, moderate, and severe effects (complete reversal of male phenotypes to female phenotypes), respectively. The concentrations chosen for the current study were based upon their ability to elicit effects on phenotype, but the least dose was only 2-fold greater than maximal estrogen equivalent concentrations measured in surface water of rivers in the United States (~0.5 µg estrogen equivalents/L) (Kolpin et al. 2002a, Kolpin et al. 2002b).

5.3. Materials and methods

5.3.1. *Rana sylvatica*

Before research commenced, all experimental procedures were approved by the University Committee on Animal Care and Supply (UCACS) at the University of Saskatchewan (Protocol #20100036). Collection of *R. sylvatica* egg masses for scientific research was approved by the Saskatchewan Ministry of Environment (Permit #10FW059). On April 8, 2010, 6 egg masses of *R. sylvatica* were collected from a communal deposition site from a pond in a relatively pristine non-agricultural area near Saskatoon, SK, Canada. Egg masses were immediately transferred to the Aquatic Toxicology Research Facility at the University of Saskatchewan and acclimated to laboratory conditions (~19 °C; light:dark cycle 16:8) and water

(filtered City of Saskatoon municipal water). Eggs began hatching on April 12, 2010, and most larvae were free-swimming on April 15, 2010, at which point exposure to EE2 was initiated.

Healthy larvae (15 per tank) were placed into 6 L of laboratory water with the appropriate nominal concentration of EE2 (1, 10, or 100 µg/L; Sigma, Oakville, ON, Canada) dissolved in an ethanol carrier (Commercial Alcohols 95% ethyl alcohol, Toronto, ON, Canada). The final concentration of ethanol in treatment tanks, including solvent controls, was 0.0025%. All treatments were replicated in triplicate tanks. Tadpoles were fed *ad libitum* daily with a slurry of Nutrafin Flake Food and Nutrafin Max Spirulina Flakes (Rolf C. Hagen, Montreal, QC, Canada).

Each day, a 50% static water renewal was performed on each tank. Briefly, half of the test solution in each tank was removed and replaced with fresh water. Then, half of the nominal concentration of EE2 dissolved in ethanol was added to each tank. Basic water quality measurements (temperature, DO, pH, conductivity) were collected daily with an YSI Quatro Multi-Parameter probe (Yellow Springs, OH, USA). Mortality of tadpoles was recorded daily. Concentrations of ammonia nitrogen, nitrate nitrogen, and nitrite nitrogen were monitored weekly with Lamotte colorimetric kits (Chestertown, MD, USA).

5.3.2. Analysis of 17α-ethynylestradiol concentrations

Concentrations of EE2 in exposure water were monitored periodically during the experiment via high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) to validate that expected nominal values were approximated. EE2 was quantified as described elsewhere (Chang et al. 2010). Briefly, samples of whole water were spiked with a deuterated internal EE2 standard (C/D/N Isotopes, Inc., Pointe-Claire, QC, Canada), extracted two times with hexane and then concentrated under nitrogen. Dried organic extracts were derivatized by use of dansyl chloride, re-extracted with hexane, dried under nitrogen, and then reconstituted in acetonitrile. Analytical detection was conducted by use of an Agilent 1200 series HPLC system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole MS/MS system (PE Sciex, Concord, ON, Canada). Both the LC and the mass spectrometer were controlled by AB Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA, USA).

5.3.3. Termination of exposure and determination of phenotypic sex

When *R. sylvatica* individuals reached metamorphic climax, they were subjected to euthanasia by use of an overdose of MS-222 (Sigma). Individuals that failed to reach metamorphic climax by 108 d post-collection were subjected to euthanasia at that time. For individuals that metamorphosed, the number of days taken to reach metamorphic climax was recorded. Each animal was weighed, measured, and gross phenotypic morphology was determined with a dissecting microscope (Olympus SZ40, Center Valley, PA, USA). Based upon the morphology of the gonads, each animal was assigned a phenotype of normal male, normal female, or abnormal phenotype. Livers were excised, flash frozen in liquid nitrogen, and stored at -80 °C until molecular analyses were performed. Then, the entire animal, including the gonads inside the body cavity, was placed into 10% formalin and fixed for at least 24 hr.

After proper fixation, gonads were re-observed for phenotype and photographed with a digital camera (Carl Zeiss AxioCam ICc3, Toronto, ON, Canada) attached to the dissecting microscope. Then, the gonad-kidney complex was removed, placed into a tissue cassette, and processed in a MVP1 Modular Vacuum Processor (Instrumentation Laboratory, Bedford, MA, USA) by use of standard histological protocols. Gonads were embedded in paraffin blocks and serially sectioned at 7 µm intervals. Tissue sections were placed onto a 37 °C water bath and then picked up on Superfrost Plus slides (ThermoScientific, Pittsburgh, PA, USA). Slides were allowed to dry on a slide warmer overnight and then stored in slide boxes until staining. For each individual, the entire gonad or at least three slides with gonad sections, including two slides from the apices of the gonad and one slide from the medial gonad, were stained with hematoxylin and eosin by use of standard protocols. After staining, sections were covered with cover slips and Microkitt xylene-based mounting medium (Serum International, Inc, Laval, QC, Canada) and allowed to dry for at least 24 hr.

Gonadal histology was evaluated using a Carl Zeiss Axio Observer.Z1 microscope equipped with a digital camera (Carl Zeiss AxioCam ICc1) and interfaced to a computer. Representative images were recorded and saved using Axiovision LE 4.7.2 software (Carl Zeiss). Slides were coded so that the observer was blinded to the treatment group that the individual belonged to. Each slide was examined for the presence of male and female tissues, and it was assessed whether those tissues were of normal morphology or not (Ankley et al. 2006).

Individuals were classified as male, female, mixed sex, or abnormal male (Hecker et al. 2006). Briefly, normal females were characterized by the presence of ovaries filled with previtellogenic and/or vitellogenic oocytes. Normal males were characterized by the presence of continuous testes filled with spermatocysts. Mixed sex individuals were characterized by gonads containing both testicular and ovarian tissues. Abnormal males were characterized by a lack of spermatocysts and/or abnormal shape of testes.

5.3.4. *Illumina* sequencing, RNA extraction, complimentary DNA synthesis, and quantitative polymerase chain reaction

Little information exists on the sequence of the genome or transcriptome of *R. sylvatica*. Before sequencing the *R. sylvatica* transcriptome for the current study, attempts were made to use sequence similarity among other species of amphibian to design primers for genes of interest for *R. sylvatica*. However, due to the phylogenetic status of *R. sylvatica* as an out-group among the North American ranid frogs (Frost et al. 2006), these attempts were largely unsuccessful and did not yield useful primers. Once a *de novo* approach to sequencing and assembling the *R. sylvatica* transcriptome was used, every set of primers that was designed using the transcriptome was suitable for quantitative polymerase chain reaction (qPCR) applications.

To establish a transcriptome for *R. sylvatica*, a 10 µg pooled sample of *R. sylvatica* RNA (2 µg RNA from livers of 5 individual frogs, both control frogs and frogs exposed to EE2 included) was sequenced on an *Illumina* HiSeq 2000 sequencer (*Illumina*, San Diego, CA, USA) at the Donnelly Sequencing Centre at the University of Toronto. Complimentary DNA (cDNA) libraries for sequencing were prepared by use of an mRNA-Seq Sample Prep Kit (*Illumina*, San Diego, CA, USA) according to the manufacturer's protocol and were sequenced as 75bp single-end reads. The raw sequences were filtered by quality, trimmed, and assembled into longer sequences, called "contigs", based on their similarities by use of a *de novo* assembly performed by CLC Genomics Workbench V 5.1 software (CLC Bio, Aarhus, Denmark). The sequence of each contig was then compared to the sequence database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) by use of Blast2GO software (Cosena

et al. 2005, Götz et al. 2008). Sequences of genes that were of interest were used to develop primers for qPCR (Table 5.1).

For qPCR, total RNA was isolated from livers of *R. sylvatica* by use of an RNeasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. RNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Primers were designed for genes of interest (Table 5.1) based on sequences from the *Illumina* analysis by use of Primer3 software (Rozen and Skaletsky 2000). Primers were designed to amplify PCR products between 100 and 150 base pairs long by use of standard parameters. Forward and reverse DNA oligos were purchased (Invitrogen, Burlington, ON, Canada). For qPCR applications, template cDNA was synthesized from 0.5 µg of *R. sylvatica* liver RNA by use of a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol.

To check primer design, PCR products were amplified for each primer set from undiluted cDNA with a Taq PCR Core Kit (Qiagen) used according to the manufacturer's protocol and a Mycycler PCR machine (Biorad, Mississauga, ON, Canada). PCR products were separated on a 1% agarose gel (Agarose PCR Plus, EMD Chemicals Inc., Gibbstown, NJ, USA) in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) with SYBR Safe DNA Gel Stain (Invitrogen). Gels were run at 90V for 1 hr, and images of gels were captured on a Versadoc MP4000 imager (Biorad) by use of visible blue light illumination. Primer sets were evaluated on the basis of presence and size of PCR product.

For all validated primer sets, efficiency curves were constructed to determine their suitability for qPCR. Briefly, for each primer set, qPCR was performed on a standard curve consisting of dilutions of cDNA. The standards were undiluted, 1:5, 1:25, 1:125, 1:625, and 1:3125 dilutions of a pool of 5 random *R. sylvatica* liver cDNA samples using a Quantitect SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol. For those standard curves with a coefficient of determination (R^2) of at least 0.99 and efficiencies of 1.9-2.1, where efficiency=$10^{(-1/\text{slope of standard curve})}$, quantification of abundances of transcripts by real time qPCR was performed with liver cDNA samples from individuals exposed to EE2. In total, qPCR was performed on 48 samples of cDNA for each target gene. Specifically, 2 male and 2 female frogs from each triplicate tank from the solvent control and 1 µg EE2/L treatments, for a total of 6 male and 6 female frogs per treatment were utilized. In addition, 2 female frogs from each

triplicate tank from the 10 and 100 µg EE2/L treatments, for a total of 6 female frogs per treatment, were analyzed. No males from these treatments were used since there were too few for an adequate sample size. All mixed sex frogs, 6 per treatment from the 1 and 10 µg EE2/L treatments, were analyzed. There were not enough mixed sex frogs from the 100 µg EE2/L treatment for an adequate sample size.

To perform qPCR, cDNA from each individual liver was diluted 1:5, and a Quantitect SYBR Green PCR Kit (Qiagen) was used according to the manufacturer's protocol. Briefly, a separate 50 µL PCR reaction consisting of SYBR Green master mix, gene-specific primers, nuclease free water, and cDNA was prepared for each cDNA sample and primer pair. Then, duplicate 20 µL reactions were transferred to a 96-well PCR plate. The qPCR was performed in an ABI 7300 Real-Time PCR System (Applied Biosystems, Burlington, ON, Canada). The PCR reaction mixture was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was as follows: denature for 15 sec at 95 °C and extension for 1 min at 60 °C for a total of 40 PCR cycles. The qPCR cycle was followed by a dissociation step to validate that all cDNA samples amplified only a single product. For each target gene, abundance of transcripts was quantified according to the Mean Normalized Expression (MNE) method of Simon (2003) using ribosomal protein L8 (*rpl8*) as a reference gene.

5.3.5. Statistics

Statistical tests were performed with IBM SPSS 19 software (IBM, Armonk, NY). Treatment means are expressed as mean ± S.D. for all data except qPCR data, which are expressed as mean ± S.E. Statistical significance was defined as $p \leq 0.05$. Where appropriate, data were subjected to tests of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) to determine whether to apply parametric or nonparametric statistical tests. Mortality and percent of tadpoles reaching metamorphic climax were analyzed with Pearson Chi-Square tests to determine differences among treatments. The mean number of days to metamorphosis was determined for each treatment using a survival analysis to allow inclusion of animals that failed to reach metamorphic climax during the experiment. The data for mean number of days to metamorphic climax, mass of frogs at metamorphic climax, and length of

frogs at metamorphic climax were analyzed with ANOVA with post-hoc Tukey's tests to determine differences among treatment means. Data for phenotypic sex were analyzed with Fisher's Exact test because values for some phenotype categories were less than 5 in some treatments. Due to a significant p-value for the Fisher's Exact test, post-hoc analysis of the phenotypic sex data was completed by comparing the standardized residual for each phenotype category in each treatment to a critical value (α=0.05, critical value=±1.96; α=0.01, critical value=±2.58) to help determine which phenotype categories drove the significant Fisher's Exact test. Where the standardized residual exceeded the critical value, the category was deemed significant (i.e. standardized residual of greater than ± 1.96 was significant with $p<0.05$). The EC_{50} values for feminization of genetic males and partial feminization of genetic males by EE2 were calculated using Probit analysis. Data for abundances of transcripts of genes of interest all deviated from normality and/or homogeneity of variances, so they were analyzed with nonparametric Kruskal-Wallis tests to determine differences among treatments. Where the p-value of the Kruskal-Wallis test was significant, post-hoc testing was performed using Mann-Whitney U tests to compare each treatment group to the control female group.

5.4. Results

5.4.1. Water quality and validation of 17α-ethynylestradiol concentrations

Water quality variables over the course of the experiment were all within an acceptable range for culture of *R. sylvatica*. Average values were temperature of 18.9 ± 0.1 °C, conductivity of 0.39 ± 0.01 mS/cm, 7.3 ± 0.1 mg dissolved oxygen/L, pH of 7.9 ± 0.1 standard units, and 0.44 ± 0.05 mg nitrate/L. Nitrite was detected in 8% of samples at concentrations of 0.02 mg nitrite/L, which was the least concentration of nitrite detectable by the kit used; the remaining 80% of samples did not have detectable concentrations of nitrite (<0.02 mg nitrite/L). Ammonia was detected in 20% of samples at concentrations of 0.02 mg ammonia/L; the remaining 80% of samples did not have detectable concentrations of ammonia (<0.02 mg ammonia/L).

The limit of quantification (LOQ) for the LC-MS/MS results presented for the current study was 0.02 µg EE2/L. Within this limit, EE2 was not detected at any point during the experiment in control or solvent control tanks. Nominal concentrations of EE2 treatments were 1, 10, or 100 µg EE2/L, and actual concentrations were 1.08 ± 0.14, 9.55 ± 1.87, or 80.90 ± 8.87 µg EE2/L immediately following water changes, respectively. To determine whether concentrations of EE2 remained stable over the 24 hr period between water renewals, concentrations were also determined just prior to water change. After 24 hr, average concentrations decreased to 0.78 ± 0.02, 8.16 ± 0.55, and 77.93 ± 1.25 µg EE2/L in the 1.08, 9.55 and 80.9 µg EE2/L treatments, respectively. These values correspond to $t_{1/2}$ values of 2.1, 4.4, and 18.5 d, respectively. Since concentrations of EE2 just prior to water change were not monitored as often as concentrations immediately following water change, validated concentrations of EE2 after water change are used hereafter to designate treatment groups.

5.4.2. Mortality, percent of tadpoles reaching metamorphic climax, days to metamorphic climax, and mass and length of frogs at metamorphic climax

Although water quality variables were similar among treatments throughout the experiment, *R. sylvatica* from the control tanks displayed significant biological differences from those in the solvent control tanks. In general, control individuals were slower to reach metamorphic climax and had less mass and length. The reason for these differences is unclear, but could have been related to colonization of tank surfaces with biofilm microorganisms after addition of the ethanol solvent. During the exposure, solvent control and treatment tanks grew visibly more biofilm than control tanks, which might have provided the tadpoles in these tanks with a source of palatable food not available to control individuals. Because of the differences between control and all other tanks, the control tanks were eliminated from all further statistical analyses, and treatment effects were based upon comparisons to solvent controls.

One replicate tank from the 80.9 µg EE2/L treatment suffered relatively great mortality compared to other tanks in the experiment due to a fungal infection that affected some tadpoles during early larval development. For this reason, there were fewer tadpoles present in this tank, which led to differences in growth parameters and time to metamorphosis for frogs from this

tank compared to the other 80.9 µg EE2/L treatment tanks. To avoid bias introduced by the atypical mortality and unequal numbers of tadpoles in each tank, the affected tank was removed from analyses of mortality, percent of tadpoles reaching metamorphic climax, time to metamorphic climax, and mass and length of frogs at metamorphic climax.

Over the course of the experiment, there were no significant differences among treatments in mortality of tadpoles (Pearson Chi-Square, p=0.126). Mortality ranged from 3-10% (Figure 5.1). Although there is no standardized guideline for acceptable mortality rates in controls for the type of experimental design utilized in the current study, mortality was at or less than the guidance standard set for a valid study by the US Environmental Protection Agency (≤10%) for the 21-day Amphibian Metamorphosis Assay utilizing *X. laevis* (USEPA 2011). At the conclusion of the experiment, not all tadpoles had reached metamorphic climax. The percentage of tadpoles that reached metamorphic climax was 84, 77, 84, and 95% in the solvent control, 1.08, 9.55, and 80.9 µg EE2/L treatments, respectively. There were no significant differences among treatments in the proportion of tadpoles that reached metamorphic climax (Pearson Chi-Square, p=0.174).

A survival analysis was used to evaluate time to reach metamorphic climax in a manner that could include those individuals that failed to reach metamorphic climax during the experiment. To determine mean time to reach metamorphic climax in each treatment, an analysis of survival was completed for each replicate tank. Replicate values were then averaged. The mean number of days required to reach metamorphic climax ranged from 91-95 d inclusive of all treatments (Figure 5.2). There were no significant differences among treatments in the number of days required to reach metamorphic climax (ANOVA, p=0.156).

The mean mass of frogs at metamorphic climax was 0.69 ± 0.03 g in the solvent control group, 0.71 ± 0.05 g in 1.08 µg EE2/L, 0.65 ± 0.04 g in 9.55 µg EE2/L, and 0.78 ± 0.03 g in 80.9 µg EE2/L (Figure 5.3). There were no statistically significant differences in mass among treatments (ANOVA, p=0.052). The mean length of *R. sylvatica* at metamorphic climax was 17.0 ± 0.2 mm in the solvent control group, 17.1 ± 0.3 mm in 1.08 µg EE2/L, 16.8 ± 0.4 mm in 9.55 µg EE2/L, and 17.6 ± 0.5 mm in 80.9 µg EE2/L (Figure 5.3). There were no significant differences in length among treatments (ANOVA, p=0.147).

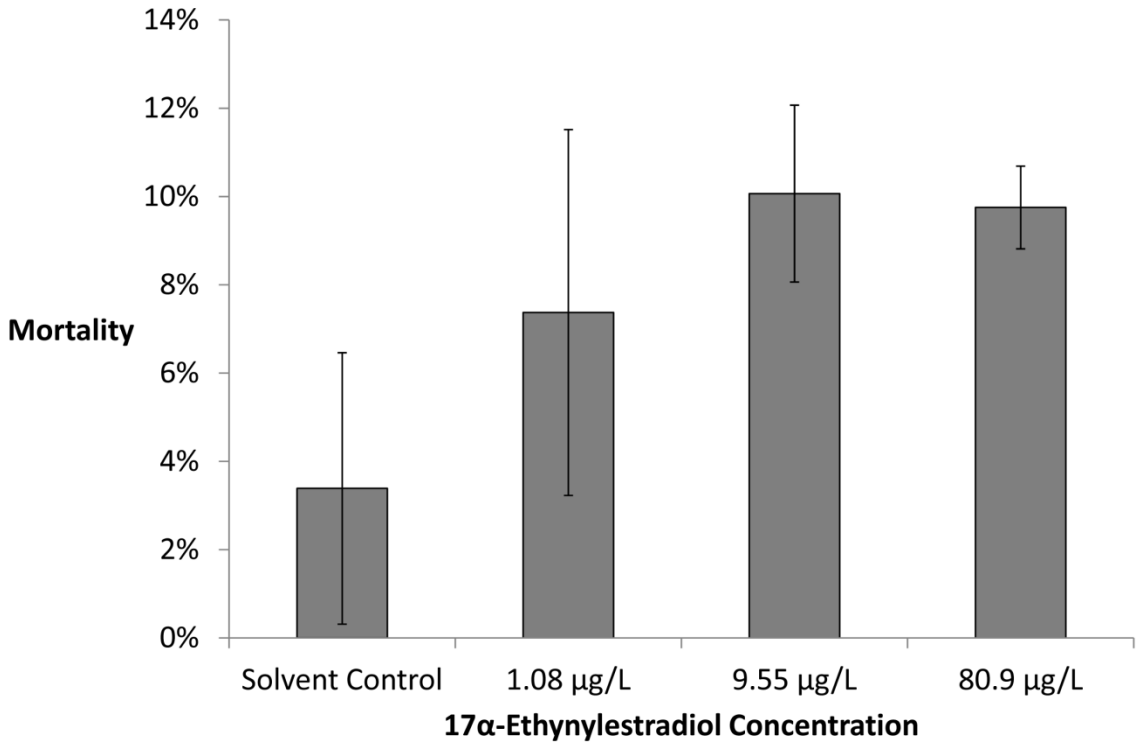

Figure 5.1: Mortality of *Rana sylvatica* tadpoles exposed to 17α-ethynylestradiol during the larval period. Mortality presented as mean ± S.D. (*n*=3 replicate tanks for solvent control, 1.08, and 9.55 µg/L treatments, *n*=2 replicate tanks for 80.9 µg/L treatment). There were no differences among treatments in the proportion of tadpoles that died during the experiment (Pearson Chi-Square, p=0.126).

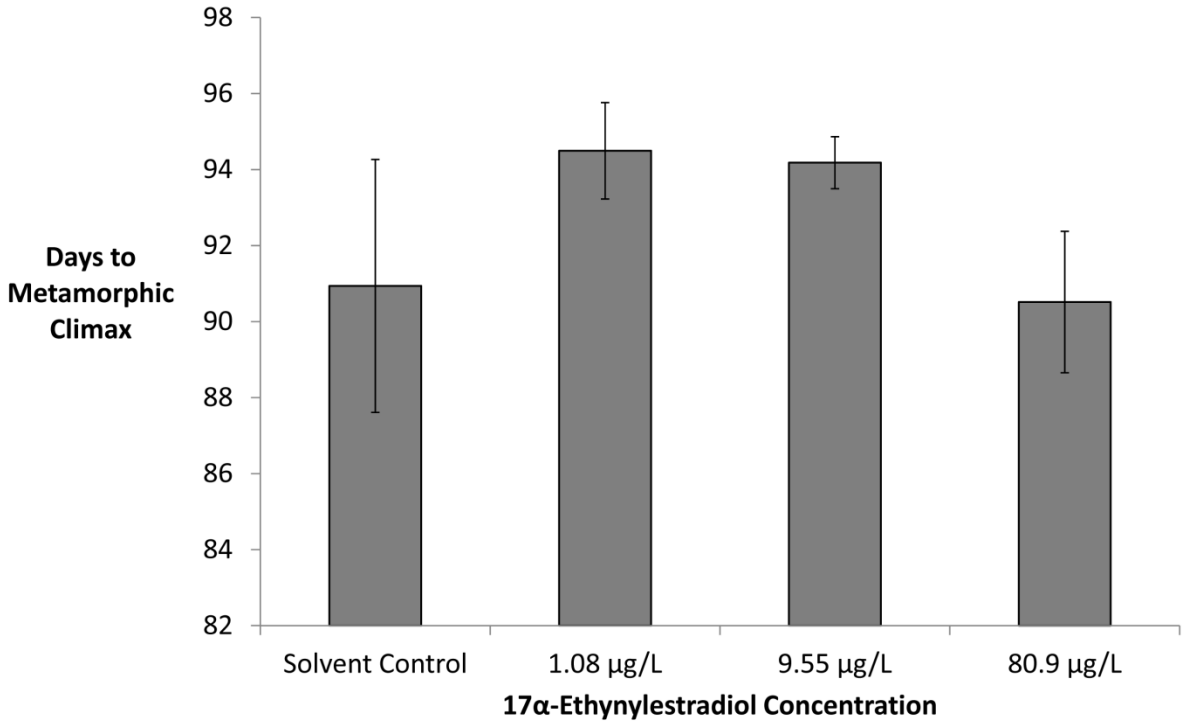

Figure 5.2: Mean number of days to reach metamorphic climax of *Rana sylvatica* tadpoles exposed to 17α-ethynylestradiol during the larval period. Data are presented as days to metamorphic climax ± S.D. (*n*=3 replicate tanks for solvent control, 1.08, and 9.55 µg/L treatments, *n*=2 replicate tanks for 80.9 µg/L treatment). There were no differences among treatments in the time required to reach metamorphic climax (ANOVA, p=0.156).

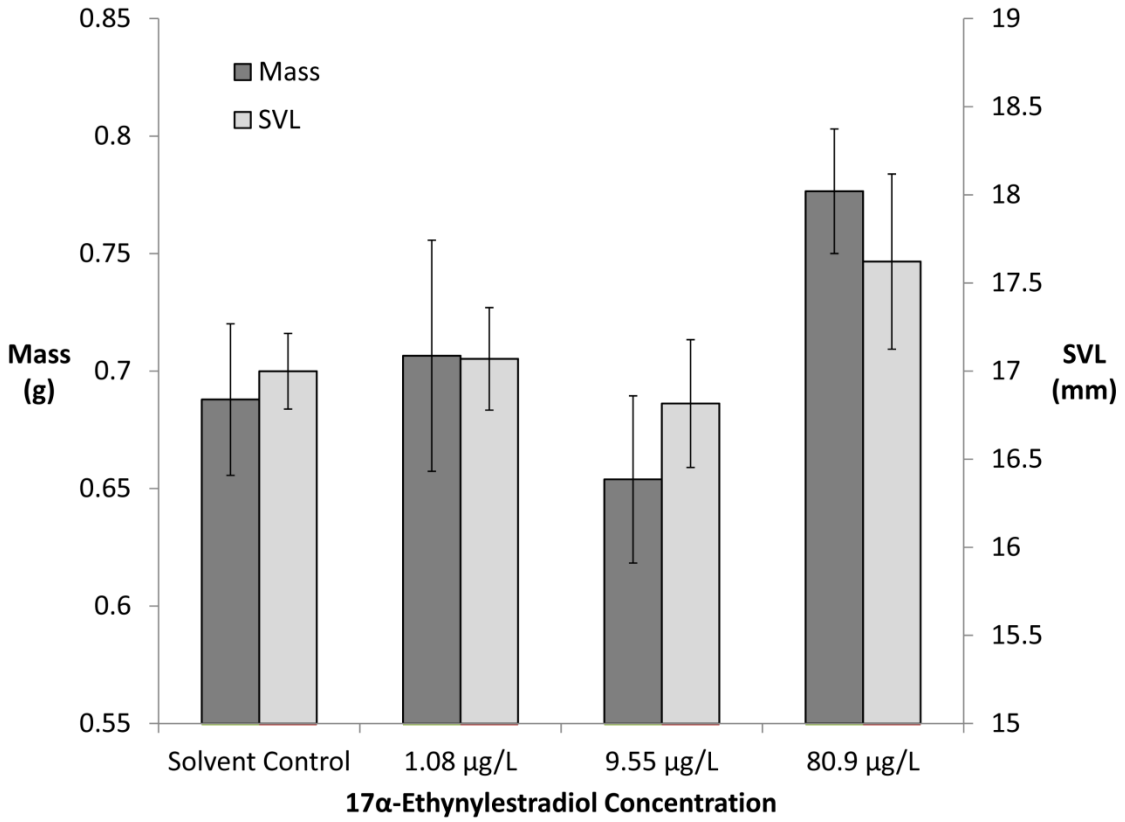

Figure 5.3: Mean mass and snout-vent-length (SVL) at metamorphic climax of *Rana sylvatica* exposed to 17α-ethynylestradiol during the larval period. Data are presented as mean ± S.D. (*n*=3 replicate tanks for solvent control, 1.08, and 9.55 µg/L treatments, *n*=2 replicate tanks for 80.9 µg/L treatment). There were no differences among treatments in the mass or length of wood frogs at metamorphic climax (ANOVA, p=0.052 for mass and p=0.147 for length).

5.4.3. Phenotypic sex ratios

All individuals were assigned to one of 4 phenotype categories based upon gross and histological analyses: female, male, abnormal male, or mixed sex (Figures 5.4 & 5.5). Phenotypic females were classified as possessing histologically normal ovarian tissue. Phenotypic male frogs from all treatments were classified as either normal males with normal testicular tissue or as abnormal males with abnormal testicular tissue. Abnormal males were characterized by atypical morphology of the gross testicular structure or by the absence of spermatocysts within the testis. Individuals with both testicular and ovarian tissue were classified as mixed sex.

There were statistically significant differences in the proportion of male, abnormal male, mixed sex, and female phenotypes among treatments (Fisher's Exact test, $p<0.0001$; Figure 5.6). Normal males accounted for 41%, 24%, 2.5%, and 0% of all individuals in the solvent control, 1.08, 9.55, and 80.9 µg EE2/L treatments, respectively. Statistically, normal males were over-represented in the solvent control treatment ($p<0.01$) and under-represented in the 8.55 and 80.9 µg EE2/L treatments ($p<0.05$). Abnormal males accounted for 5%, 3%, 0%, and 0% of all individuals in the solvent control, 1.08, 9.55, and 80.9 µg EE2/L treatments, respectively. There were no statistically significant differences in the proportion of abnormal males among treatments. Mixed sex individuals accounted for 0%, 16%, 15%, and 3% of all individuals in the solvent control, 1.08, 9.55, and 80.9 µg EE2/L treatments, respectively. Phenotypic females accounted for 55%, 58%, 82%, and 97% of all individuals in the solvent control, 1.08, 9.55, and 80.9 µg EE2/L treatments, respectively. Although the standardized residuals for proportions of mixed sex individuals and phenotypic females in each treatment did not meet the probability of Type I Error established for statistical significance, there were some treatments in each category that trended toward significance. In general, mixed sex individuals were under-represented in the solvent control treatment, and females were over-represented at the two greatest concentrations of EE2.

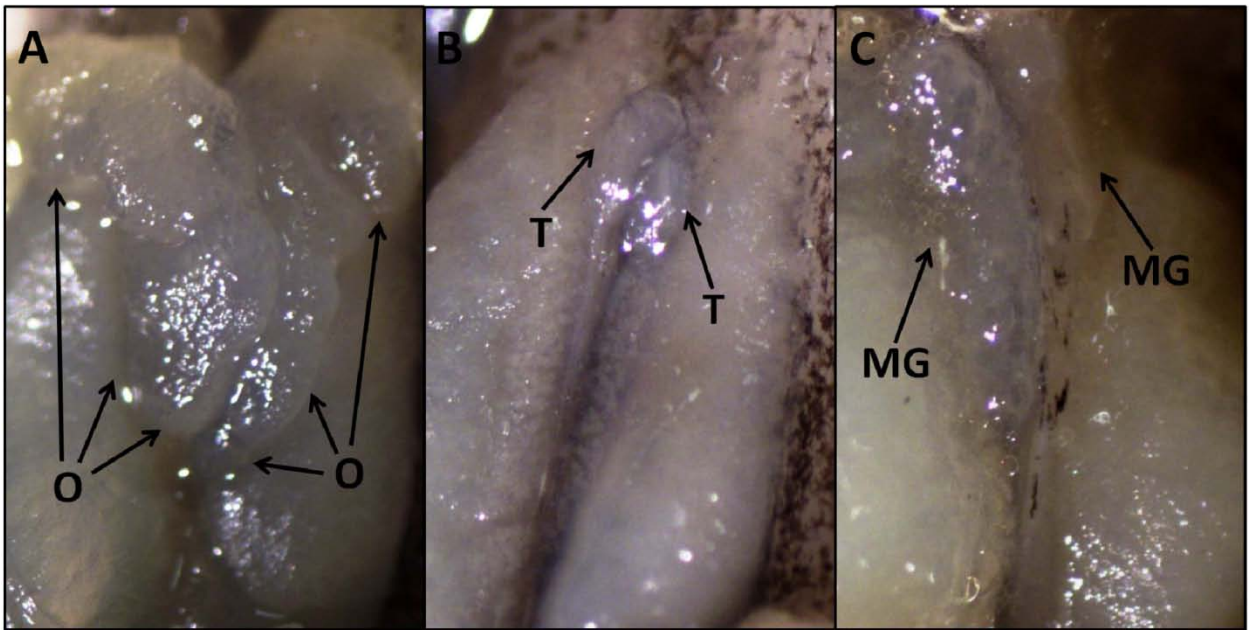

Figure 5.4: Gross gonadal morphology of *Rana sylvatica* exposed to 17α-ethynylestradiol during the larval period. Representative morphological pictures of three of the phenotype categories utilized to classify gonads: A) Normal female with two normal ovaries (O); B) Normal male with two normal testes (T); C) Mixed sex individual with two mixed sex gonads (MG).

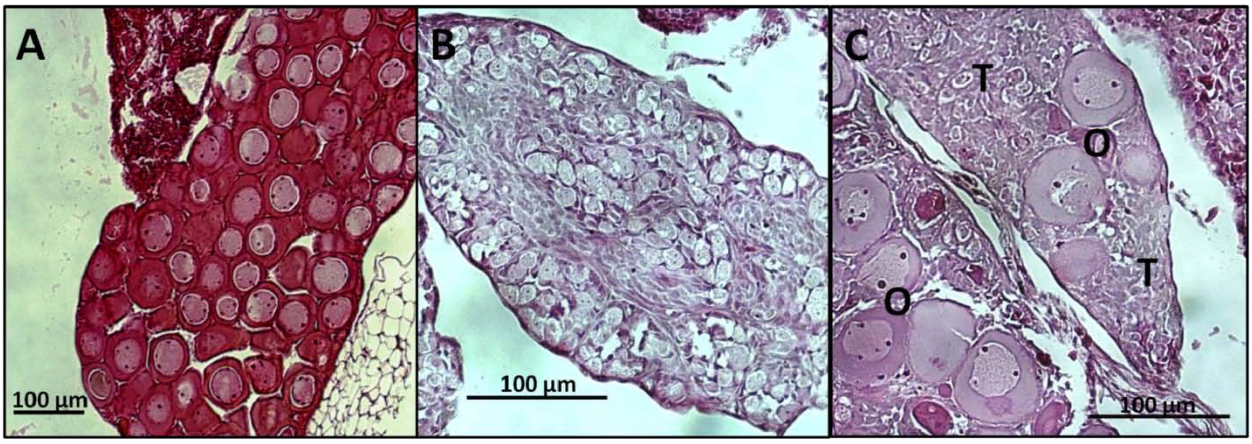

Figure 5.5: Gonadal histology of *R. sylvatica* exposed to 17α-ethynylestradiol during the larval period. Representative images of three of the phenotype categories utilized to classify gonads: A) Normal female ovary; B) Normal male testis; C) Mixed sex gonads with both testicular (T) and ovarian (O) tissues.

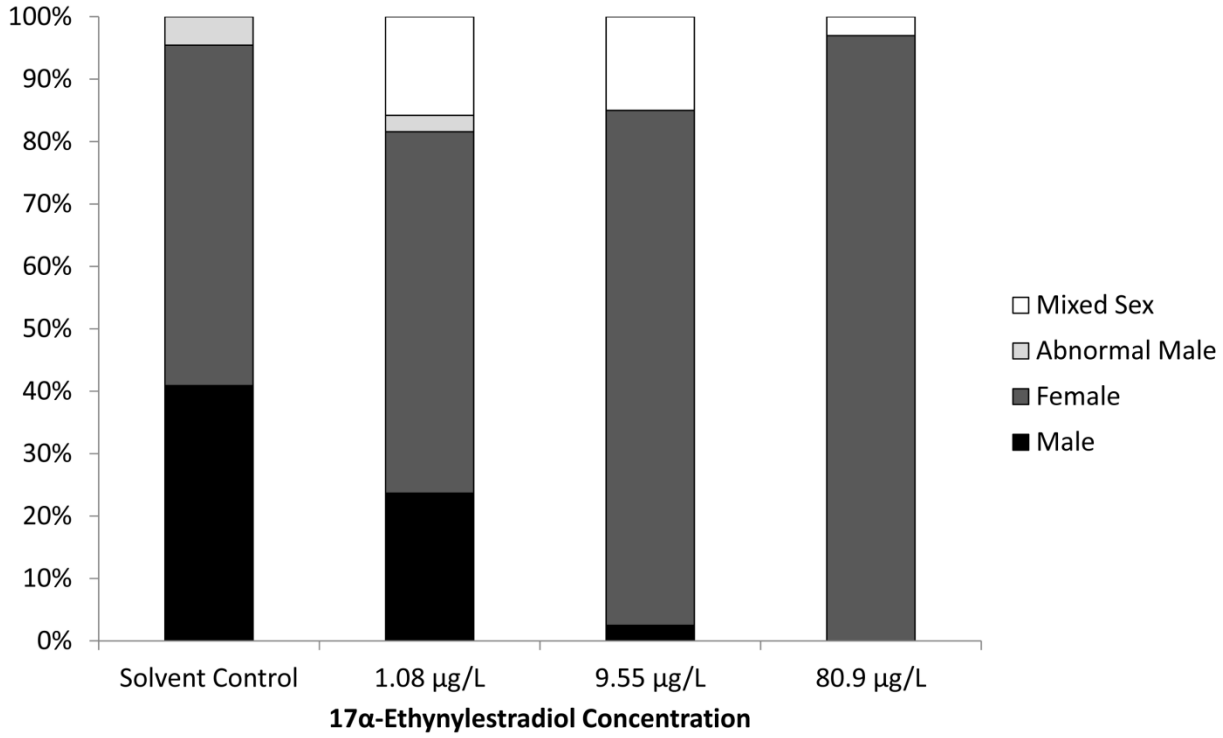

Figure 5.6: Phenotypic sex ratios of *Rana sylvatica* exposed to 17α-ethynylestradiol during the larval period. Data are presented as the overall percentage of individuals in each phenotype category (male, female, abnormal male, mixed sex) (*n*=33-44 *R. sylvatica* per treatment group). There were statistically significant differences among treatments in sex ratios (Fisher's Exact test, $p<0.0001$). Increasing EE2 concentrations led to a lesser proportion of *R. sylvatica* developing with male phenotypes, and a greater proportion of *R. sylvatica* developing with mixed sex and female phenotypes.

5.4.4. EC_{50} for feminization of genetic males

The EC_{50} for complete feminization of genetic males was calculated based upon the number of phenotypic females present in EE2 treatments normalized to the number of phenotypic females present in the solvent control treatment. Mixed sex individuals were not considered to be completely feminized. Using Probit analysis, the EC_{50} for feminization of presumed genetic males was 7.7 µg EE2/L, and the 95% confidence interval for the EC_{50} was 3.8-15.1 µg EE2/L.

The EC_{50} for partial feminization of genetic males was calculated based on the combined number of phenotypic females and mixed sex individuals present in EE2 treatments normalized to the number of phenotypic females present in the solvent control treatment. For this analysis, all mixed sex individuals were presumed to be genetic males since previous studies with *X. laevis* have shown that the sexual differentiation of only genetic males is impacted by exposure to EE2 (Tompsett et al. 2012). Using Probit analysis, the EC_{50} for partial feminization of presumed genetic males was 2.3 µg EE2/L, and the 95% confidence interval for the EC_{50} for partial feminization was 0.5-5.3 µg EE2/L.

5.4.5. Abundances of transcripts of genes of interest

The abundances of transcripts of 8 genes that are involved in steroid signaling and metabolism, cholesterol biosynthesis, or vitellogenesis (Table 5.1) were measured in the livers of *R. sylvatica* using qPCR. For 3 of the 8 genes, including vitellogenin A2 (*vtga2*), high density lipoprotein binding protein (*hdlbp*), and 7-dehydrocholesterol reductase (*dhcr7*), there were significant differences among treatments in the abundances of transcripts (Kruskal-Wallis, p=0.001, 0.028, and 0.013 for *vtga2*, *hdlbp*, and *dhcr7*, respectively). For *vtga2*, abundances of transcripts were significantly greater than abundances of solvent control females, ranging from 200-280-fold greater, in all groups exposed to EE2 (Figure 5.7). For *hdlbp*, abundances of transcripts were significantly greater than abundances of solvent control females, ranging from 1.8-3.1-fold greater, in female and mixed sex individuals exposed to 1.08 µg EE2/L, female and mixed sex individuals exposed to 9.55 µg EE2/L, and female individuals exposed to 80.9 µg

EE2/L (Figure 5.8). For *dhcr7*, abundances of transcripts were significantly greater than abundances of solvent control females in mixed sex individuals exposed to 1.08 and 8.81 µg EE2/L by 4.9-fold and 4.3-fold, respectively (Figure 5.9).

Table 5.1: *Rana sylvatica* primer sets for liver-specific genes expected to be impacted by estrogen exposure and a reference gene, *rpl8*

Gene Identity	Abbreviation	Function[a]	Forward primer	Reverse primer
Estrogen sulfotransferase	*est*	SM	TTCCACAACATGGTGAAAGC	GATCATGCCAGGAACCGTAG
Liver X receptor	*nr1h2*	SM	AATGCAGAGAGGCTGGAATG	CTAGGTGGAGCCAGAACTGC
Liver receptor homolog-1	*nr5a2*	SM	CCCTTCCCCCAACAGACTAT	GGTGTAGGGATCCGGGTACT
Androgen receptor	*ar*	SM	AGTGGCTGAGGAGGACAAGA	GTGCAGGGCTGCTCTTTATC
7-dehydrocholesterol reductase	*dhcr7*	CB	CCAGGCTATGTTGGAGGTGT	GGTTGGAGAGAACCAGTGGA
Estrogen receptor α	*era*	VTGS	TGGCTTGAAATCCTCATGGT	AACCAAGCCTTCCACACAAC
High density lipoprotein binding protein	*hdlbp*	VGTS	CAGTGACCTGATTGCTCGAA	GGGGAATACGCACTGACACT
Vitellogenin A2	*vtga2*	VTGS	ACAGGTGTGTCCCTGGTAGC	TACCGCATAACTGGCCTTTC
Ribosomal protein L8	*rpl8*	RF	GGCTACATCAAGGGCATTGT	GATACCCTCAGCCGCAATAA

[a] SM=steroid metabolism; VTGS=vitellogenesis; CB=cholesterol biosynthesis; RF=reference

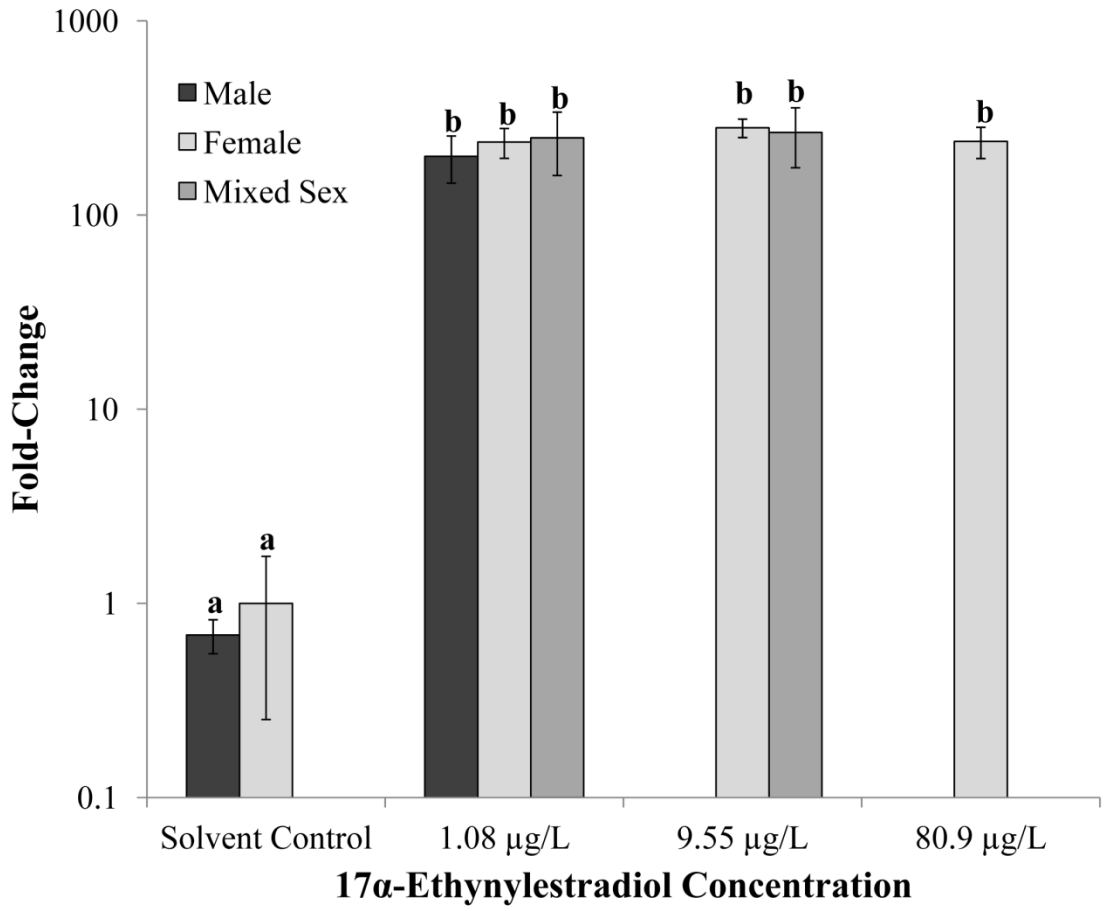

Figure 5.7: Fold-changes in abundances of transcripts of vitellogenin A2 (*vtga2*) in the livers of *Rana sylvatica* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E and are displayed on a log scale. There were significant differences in abundances of *vtga2* transcripts among treatments after exposure. Significant differences from the control group are denoted by different letters (Kruskal-Wallis, p=0.001; *n*=6 frogs in each category).

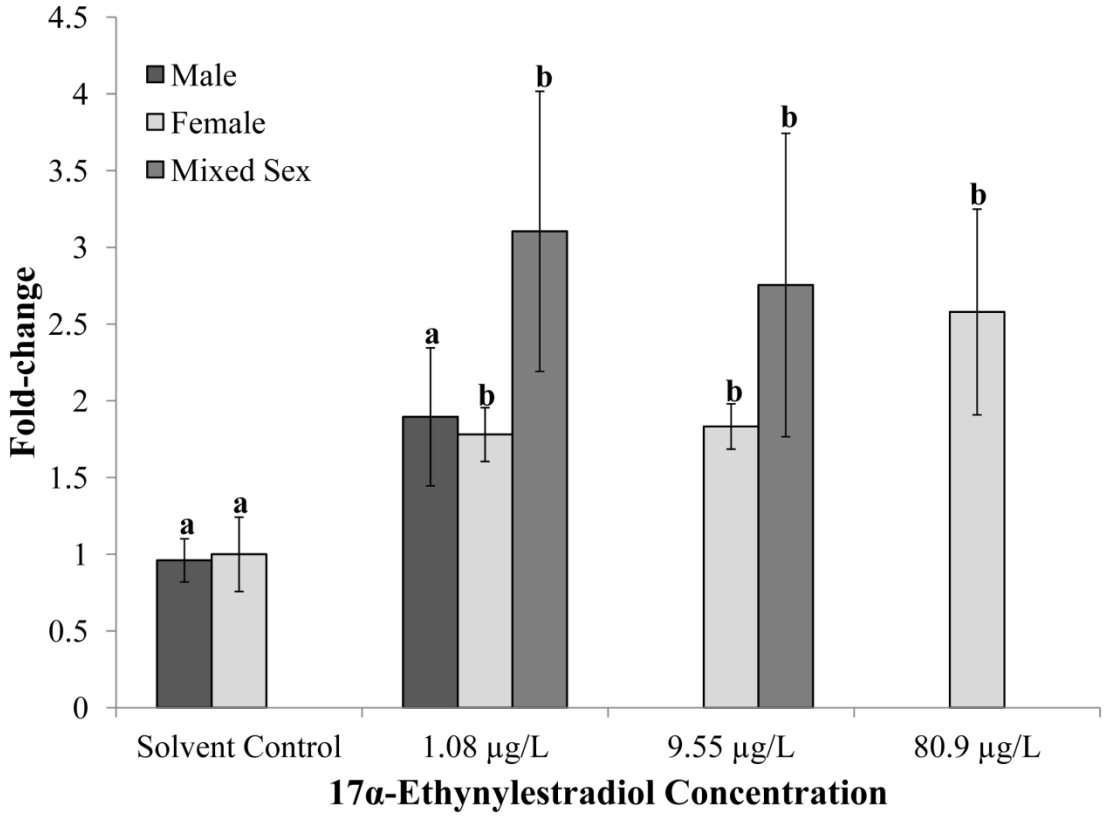

Figure 5.8: Fold-changes in abundances of transcripts of high density lipoprotein binding protein (*hdlbp*) in the livers of *Rana sylvatica* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of *hdlbp* transcripts among treatments after exposure. Significant differences from the control group are denoted by different letters (Kruskal-Wallis, p=0.028; *n*=6 frogs in each category).

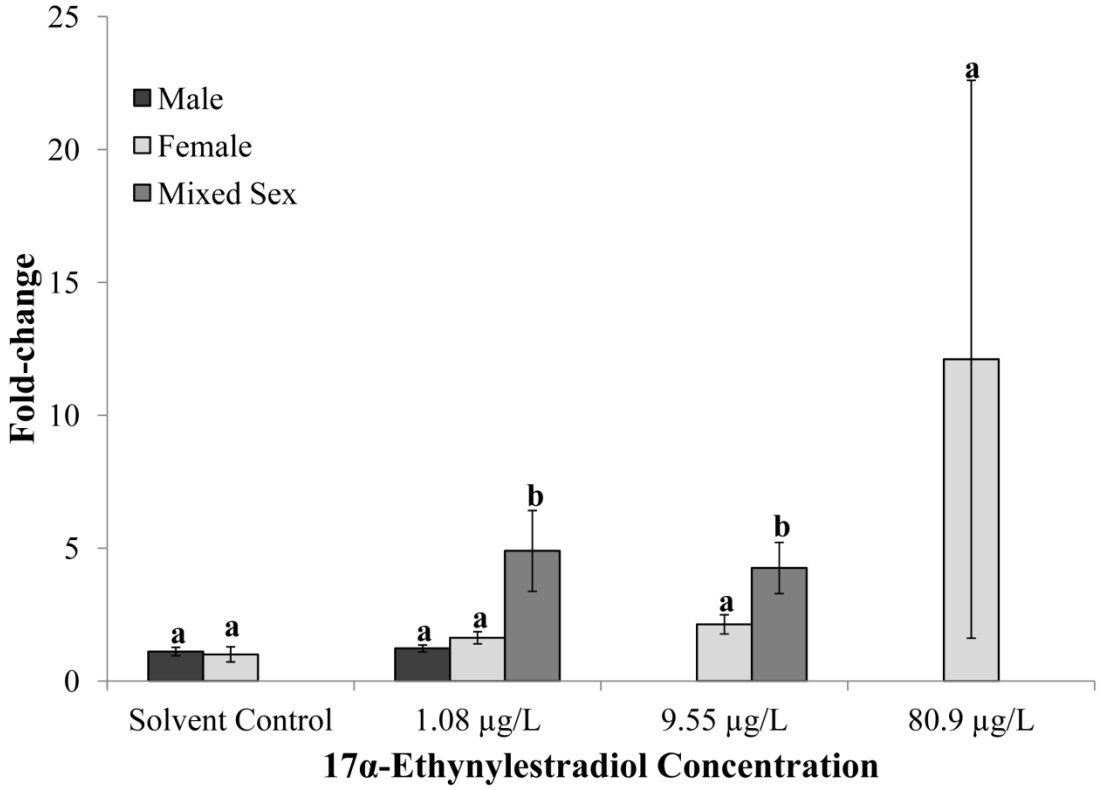

Figure 5.9: Fold-changes in abundances of transcripts of 7-dehydrocholesterol reductase (*dhcr7*) in the livers of *Rana sylvatica* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of *dhcr7* transcripts among treatments after exposure. Significant differences from the control group are denoted by different letters (Kruskal-Wallis, p=0.034; *n*=6 frogs in each category).

5.5. Discussion

5.5.1. Sexual differentiation, growth, mortality, and time to metamorphosis

Exposure to EE2 during the larval period significantly impacted phenotypic sex ratios of *R. sylvatica*. Specifically, in the current study, the proportion of phenotypic male tadpoles was greater in the solvent control treatment than the proportion of phenotypic males in the 9.55 and 80.9 µg EE2/L treatments. There was also a trend of a greater number of individuals with a mixed sex phenotype in the 1.08 and 9.55 µg EE2/L treatments compared to the solvent control treatment. Utilizing the data from the current study, the EC_{50} for complete feminization of presumed genetic male tadpoles to phenotypic females was determined to be 7.7 µg EE2/L, and the EC_{50} for partial feminization of presumed genetic male tadpoles to phenotypic females was determined to be 2.3 µg EE2/L.

Although the response of *R. sylvatica* exposed to EE2 during sexual determination and differentiation has been characterized previously (Witschi 1951, Mackenzie et al. 2003), the current study used an improved experimental design, which allowed more precise determination of the effective concentration of EE2 required to feminize presumed genetic male *R. sylvatica* tadpoles. Although EE2 is more persistent than the endogenous steroid hormone E2 (Ying et al. 2002), it has been noted to degrade during static renewal amphibian exposures. Both Witschi (1951) and Mackenzie et al. (2003) used static renewal exposure systems with renewals every 48-72 hr, and both noted that concentrations of EE2 decreased over time. In one case, actual exposure averaged about 41% of nominal concentrations (Mackenzie et al. 2003). In the current study, a 24 hr static renewal system was employed, which lessened the effect of EE2 degradation, and EE2 was found to be only 4-28% less than initially measured concentrations after 24 hr.

Using the 24 hr static renewal experimental design, the effective concentrations for feminization of presumed genetic males were found, in general, to be less than those previously reported in the literature for *R. sylvatica*. The initial work performed by Witschi (1951) indicated that there was no effect of treatment of *R. sylvatica* tadpoles with 1 µg EE2/L, partial feminization at 10 µg EE2/L, and complete feminization at 100 µg EE2/L. However, a more recent study indicated that treatment with 1 or 10 µg EE2/L did not affect the number of mixed

sex individuals or male:female sex ratios of *R. sylvatica*, although that study lacked proper replication (Mackenzie et al. 2003). The EC_{50} for complete feminization (7.7 µg EE2/L) observed in the current study is probably comparable to the effect level described by Witschi (1951), although no data were reported in that study that could be used to confirm this conclusion. However, the EC_{50} for partial feminization (2.3 µg EE2/L) is less than previously described, since Witschi (1951) found no effects of treatment with 1 µg EE2/L while 16% tadpoles exposed to 1.08 µg EE2/L in the current study developed with mixed sex phenotypes. It is probable that this difference is attributable to the more frequent water renewals used in the current study. Since none of the concentrations of EE2 tested in the current study resulted in no effects on sexual development of *R. sylvatica*, it is possible that the EC_{50} for partial feminization is even less than that reported here. For this reason, additional studies utilizing lesser concentrations of EE2 would be useful to refine this value.

In comparison with other amphibians, *R. sylvatica* exhibited average sensitivity to alterations of sexual differentiation and development induced by exposure to EE2. The Western clawed frog (*Silurana (Xenopus) tropicalis*) is more sensitive to estrogenic compounds and exhibits female-biased sex ratios after exposure to 0.06 nM EE2 (~18 ng/L) (Pettersson and Berg 2007). However, the EC_{50} for feminization by E2 of the related African clawed frog (*X. laevis*) has been shown to average 0.2 µg E2/L (Wolf et al. 2010), and a recent study with EE2 indicated that *X. laevis* might be even less sensitive than *R. sylvatica* to complete feminization by EE2, since treatment with up to 8.81 µg EE2/L did not result in phenotypic sex reversal of 50% of genetic males (Tompsett et al. 2012). Compared to other members of the Ranidae family, especially those native to North America, *R. sylvatica* has average sensitivity to phenotypic sex reversal by EE2. The leopard frog (*Rana pipiens*) showed female-biased sex ratios when exposed to 5 nM EE2 (~1.5 µg/L) from Gosner stages 27-30, although chronic exposure to the same concentration of EE2 did not alter sex ratios but resulted in a greater proportion of frogs developing mixed sex phenotypes (Hogan et al. 2008). Similarly, chronic exposure of *R. pipiens* to 1 or 10 µg EE2/L resulted in sex ratios that differed significantly from those of control frogs, mostly driven by a greater number of mixed sex frogs in treatments with EE2 (Mackenzie et al. 2003). In addition, in a field experiment that included both caged and wild-caught frogs, it was found that exposure to 5 ng EE2/L induced up to 29% of mink frogs (*Rana septentrionalis*) to develop a mixed sex phenotype, although there were no effects on sexual differentiation of the

green frog (*Rana clamitans*) (Park and Kidd 2005). Clearly, different species of amphibian differ in their sensitivity to exposure to EE2. However, in all cases, effects on sexual differentiation were found at concentrations that were either environmentally relevant, or within 2-fold of concentrations of estrogen equivalents previously measured in surface waters in the United States (Kolpin et al. 2002a, Kolpin et al. 2002b).

Although there were clear effects of exposure to EE2 on differentiation and development of gonads, there were no impacts of exposure to EE2 at concentrations of up to 80.9 µg EE2/L on other biological endpoints measured for *R. sylvatica*. There was no effect of exposure to EE2 on the mass or snout-vent-length of frogs at metamorphic climax in the current study, although previous studies had indicated that this was a possibility, at least for snout-vent length. Specifically, exposure to 10 µg EE2/L was found to significantly increase the snout-vent-length of metamorphosed frogs in a previous study, although there were no effects of EE2 on mass (Mackenzie et al. 2003). In fact, a 2-week exposure of *R. sylvatica* tadpoles to 0.5 µM EE2 (~150 µg/L) and 0.75 µM EE2 (~225 µg/L) showed that exposure to only the greater dose led to significantly increased body mass (Hogan et al. 2006). In addition, in the current study, exposure to EE2 did not impact mortality of larvae, which is not surprising considering the LC_{50} for EE2 for *R. sylvatica* is 1.89 µM EE2 (~568 µg/L) (Hogan et al. 2006).

Previous studies have indicated that potent estrogens have the potential to delay metamorphosis in a number of species of amphibians, including *R. pipiens* (Hogan et al. 2008), *R. temporaria* (Roth 1948), and *X. laevis* (Gray and Janssens 1990, Lutz et al. 2008, Tompsett et al. 2012). However, the effects of estrogens on metamorphosis are not consistent either between or within species (Pettersson and Berg 2007, Lutz et al. 2008, Wolf et al. 2010). In the current study, there was no effect of exposure to EE2 at concentrations of up to 80.9 µg EE2/L on the time required by larvae of *R. sylvatica* to reach metamorphic climax. Similarly, exposure to 1 or 10 µg EE2/L did not impact the time required to complete metamorphosis by *R. sylvatica* tadpoles previously (Mackenzie et al. 2003).

5.5.2. Abundances of transcripts of target genes in the liver

In the current study, long-term exposure of *R. sylvatica* to EE2 at concentrations within 2-fold of concentrations of estrogen equivalents previously measured in the environment significantly altered the abundances of transcripts of *vtga2, hdlbp,* and *dhcr7* in liver. Two of these transcripts, *vtga2* and *hdlbp*, are involved in vitellogenesis, while the remaining transcript, *dhcr7*, is involved in the synthesis of cholesterol. The magnitude of significant alterations in abundances of transcripts in livers from frogs exposed to EE2 ranged from +4.3-4.9-fold for *dhcr7*, +1.8-3.1-fold for *hdlbp,* and +200-280-fold for *vtga2*, and the changes were detectable after up to 100 d of constant exposure to EE2. This suggests that alterations in abundances of these transcripts might be suitable endpoints for monitoring subchronic and chronic effects of estrogens in the environment on livers of amphibians. Previous experiments, including Chapter 4 of this thesis, have demonstrated that alterations in abundances of transcripts of genes in the liver are reliable indicators of chronic exposure of amphibians to chemicals that impact endocrine homeostasis (Duarte-Guterman et al. 2010).

Although previous studies had indicated that individuals with altered phenotypes might display characteristic alterations in abundances of transcripts related to phenotype (Duarte-Guterman et al. 2010), in the current study, there was limited evidence that individuals with mixed sex and sex-reversed phenotypes manifested these differences via characteristic shifts in the abundances of transcripts of the genes quantified in this study, at least at the point of metamorphic climax. In terms of statistical differences, mixed sex individuals had greater abundances of transcripts of *dhcr7* than solvent control individuals or other individuals that were exposed to EE2, but the variability in abundances of transcripts in individuals within treatments might have masked alterations in abundance of transcripts of *dhcr7* (Figure 5.9). The lack of effects driven by phenotype might be due to the relative sexual immaturity of *R. sylvatica* at the point of metamorphic climax. Since these individuals were 1-2 years from reaching sexual maturity (Conant and Collins 1998), it is feasible that they were not at a point in development where sexually dimorphic expression of genes was possible.

Because *R. sylvatica* in the current study were chronically exposed to EE2 for up to100 d, the lack of alterations in abundances of transcripts of genes involved in metabolizing and detoxifying steroids was somewhat unexpected. The protein products of *ar, nr5a2, nr1h2*, and

especially *est*, which codes for a sulfotransferase enzyme that preferentially sulfates endogenous and exogenous estrogens, are integral to maintaining endocrine homeostasis and metabolizing and eliminating both endogenous and exogenous hormones from circulation (Kauffman 2004, Yasuda et al. 2005). It is unclear why the abundances of transcripts of these genes were unchanged, but the chronic nature of the study could have contributed, since the endocrine system is regulated via feedback loops to maintain homeostasis. Since there were no detectable differences at the transcript level, it might have been more useful to measure endpoints other than transcripts, such as enzyme activity or protein concentration, since post-transcriptional and post-translational modifications of mRNAs and proteins/enzymes can have impacts on biological activity that are not revealed by measurement of abundances of transcripts.

The *DHCR7* gene codes for the enzyme that catalyzes the formation of cholesterol from 7-dehydrocholesterol and completes the final step of cholesterol biosynthesis. In the current study, the abundance of transcripts of *dhcr7* in mixed sex individuals exposed to 1.08 and 9.55 µg EE2/L was significantly greater than the abundance in solvent control individuals. In other non-mammalian vertebrates, exposure to estrogens also affects synthesis of cholesterol. *X. laevis* livers produced greater amounts of fatty acids and cholesterol after exposure to E2 (Smith et al. 1978). In addition, in female fathead minnows (*Pimephales promelas*), exposure to EE2 increased total cholesterol in the liver after 4-8 d of exposure (Ekman et al. 2009), and concentrations of cholesterol were also greater in livers of goldfish (*Carassius auratus*) after 5 months of exposure to E2 (Sharpe and MacLatchy 2007). However, since only one transcript involved in the synthesis of cholesterol was monitored in the current study, it is unclear whether there were similar effects on concentrations of cholesterol in *R. sylvatica* or on other portions of the pathway of cholesterol biosynthesis.

The abundances of transcripts of three genes involved in the process of vitellogenesis, *era*, *hdlbp*, and *vtga2* were quantified in the current study. Of the three, abundances of transcripts of *hdlbp* and *vtga2* were altered by chronic exposure to EE2. The abundances of transcripts of *vtga2* were significantly greater in individuals from all groups exposed to EE2 than abundances in females exposed to the solvent control, and fold-changes ranged from 200-280-fold greater (Figure 5.7). Vitellogenin (VTG) is a yolk-precursor protein that is normally produced by female organisms that are preparing to generate eggs to spawn. The gene coding for VTG is present in both genetic male and female organisms, but males and juvenile

individuals do not express great quantities of it under normal circumstances. However, expression of VTG is rapidly induced in response to exposure to estrogens, even in genetic males, via regulation by an estrogen response element in the promoter region of the gene (reviewed by Rotchell and Ostrander 2003). In the current study, a positive-feedback loop where exposure to EE2 leads to greater numbers of activated estrogen-receptor complexes that can interact with the *VTGA2* promoter and induce expression of the gene readily explains the greater abundance of transcripts of *vtga2*, even in sexually immature or male individuals that would not normally express this gene. The protein product of *hdlbp* transcripts acts as a binding and stabilizing protein of *vtg* transcripts (Dodson and Shapiro 1997, Cunningham et al. 2000), and the abundances of transcripts of this gene were significantly greater than abundances in females exposed to the solvent control in female and mixed sex individuals exposed to EE2 (Figure 5.8). The HDLBP protein has been shown to increase the half-life of transcripts of *vtg* from 16 h to 500 h in cultured liver cells from *X. laevis* (Brock and Shapiro 1983). This might partially explain the relatively great fold-changes in abundances of transcripts of *vtga2* in individuals exposed to EE2. Without this stabilizing effect of HDLBP, the transcripts would be expected to have a much greater rate of turnover.

The greater abundance of transcripts of *vtga2* in the livers of *R. sylvatica* after chronic exposure to EE2 is similar to previous results documented in *X. laevis* in Chapter 4. A chronic exposure of *X. laevis* to EE2 during larval and post-metamorphic development found that the abundance of transcripts of *vtga2* were as much as 102-fold greater in frogs exposed to EE2 compared to control frogs. Using immunohistochemical localization it was determined that the *X. laevis* individuals from that study also had greater amounts of VTG protein in the kidney-gonad complexes (Tompsett et al. 2012). However, *R. sylvatica* from the current study showed no indication of the presence of additional protein in the kidney-gonad complexes upon histological examination and for this reason no attempt was made to localize VTG. The differences observed between the two species could be due to the fact that the *X. laevis* were grown for a few weeks after metamorphosis while *R. sylvatica* were sampled at metamorphic climax. *X. laevis* does not gain the ability to express the vitellogenin gene until around the point of metamorphic climax (Tata et al. 1993), so it might be that *R. sylvatica* had also just gained this ability and did not have additional time to accumulate protein that could be visualized in the

gonads and kidneys. However, further studies would need to be performed to characterize the temporal response of *R. sylvatica* vitellogenesis during exposure to EE2.

CHAPTER 6

General Discussion

6.1. Abstract

In general, exposure to 17α-ethynylestradiol (EE2) during the larval period feminized and/or demasculinized both the African clawed frog (*Xenopus laevis*) and the wood frog (*Rana sylvatica*). However, the observed responses to EE2 differed intrinsically between the two species. The EC_{50} for complete feminization for *R. sylvatica* was determined to be 7.7 µg EE2/L, but it was not possible to calculate a similar value for *X. laevis* since even the greatest concentration of EE2 tested (8.81 µg EE2/L) did not feminize 50% of genetic males. The EC_{50} values for partial feminization were determined to be 2.3 and 8.81 µg EE2/L for *R. sylvatica* and *X. laevis*, respectively. Exposure to EE2 did not have significant effects on growth or metamorphic endpoints in *R. sylvatica* but did significantly delay the completion of metamorphosis in *X. laevis*. After chronic exposure to EE2, the abundances of transcripts of 6 genes, including estrogen sulfotransferase, estrogen receptor, androgen receptor, high density lipoprotein binding protein, vitellogenin A2, and farnesyl diphosphate synthase, were evaluated in *R. syvatica* and *X. laevis* livers. From this group, abundances of transcripts of vitellogenin A2 were affected similarly in both species, with generally greater abundances in individuals exposed to EE2. Although specific responses to EE2 differed between *R. sylvatica* and *X. laevis*, both species were affected at concentrations of EE2 within the realm of environmental relevance. As such, exposure to EE2, and other estrogenic compounds, could be of concern for these two species of frog.

6.2. Introduction

Characterization of the toxicity of synthetic chemicals to amphibians is integral to understanding whether these chemicals could be of concern to populations of amphibians in the wild, especially since populations of some species of amphibians are in decline around the world (Stuart et al. 2004, Wake 2012). Although the reasons for these declines are often unclear, exposure of amphibians to contaminants in the environment has been suggested as a contributing factor to these declines in some cases (Hayes et al. 2002, Koprivnikar et al. 2006, Rohr et al. 2008a, Rohr et al. 2008b). For this reason, there is a need for additional baseline data on the

sensitivity of amphibians to a range of chemical contaminants, including pesticides, herbicides, steroid hormones, pharmaceuticals, and personal care products. One class of contaminants that is of particular environmental concern consists of chemicals with estrogenic activity, which includes both natural and synthetic estrogens and a variety of industrial products. 17α-ethynylestradiol (EE2) is a synthetic estrogen utilized in pharmaceutical oral contraceptives and is not efficiently removed from wastewater by treatment facilities (Ankley et al. 2007), so it is present in effluents released to the aquatic environment. It has been detected in the environment at concentrations of up to ~0.3 µg EE2/L and is present at median concentrations of 0.09 µg EE2/L in U.S. surface waters (Kolpin et al. 2002a, Kolpin et al. 2002b). Other studies have shown that contamination of surface waters with environmental estrogens, including EE2, is a concern worldwide (reviewed by Kidd et al. 2007). Thus, exposure of amphibians to EE2 in the environment could be of concern.

The sensitivity of some species of amphibian to exposure to exogenous estrogens, including EE2, has been described previously. Briefly, exposure to these compounds during the period of sexual determination and differentiation causes abnormal gonadal development and male-to-female reversal of phenotypic sex in a number of species (Witschi 1951, Chang and Witschi 1955, Witschi 1958, Park and Kidd 2005, Pettersson et al. 2006, Pettersson and Berg 2007, Hogan et al. 2008). Other reproductive abnormalities, including impaired spermatogenesis (Lee et al. 2005, Hu et al. 2008), abnormalities of oviduct formation (Pettersson et al. 2006), and development of mixed sex phenotypes (Park and Kidd 2005, Hu et al. 2008) have also been linked to exposure to estrogens. However, prior to the studies performed during the completion of this thesis, the specific responses of the African clawed frog (*Xenopus laevis*) and the wood frog (*Rana sylvatica*) to exposure to EE2 during the larval period had not been fully characterized or were poorly characterized. In addition, there had been few efforts (Yoshimoto et al. 2008, Okada et al. 2009) to determine the molecular effects of exposure to EE2 either during or after the period of sexual determination and differentiation in *X. laevis*, and no studies have characterized these types of effects in *R. sylvatica*.

The studies contained in this thesis were designed to characterize effects of exposure to EE2 on two species of amphibian, the African clawed frog (*X. laevis*) and the wood frog (*R. sylvatica*). To do so, two laboratory experiments were performed where tadpoles were exposed to EE2 throughout larval development and beyond the conclusion of metamorphosis in the case

of *X. laevis*. In each experiment, one species was chronically exposed to EE2, and general biological, molecular, and phenotypic effects of the exposure were evaluated. While a range of concentrations of EE2 were utilized in the studies, the *X. laevis* study evaluated two concentrations that were considered within the realm of environmental relevance (0.09 and 0.84 µg EE2/L), and the *R. sylvatica* study evaluated one (1.08 µg EE2/L). Therefore, the results of these studies can be used to hypothesize the potential effects of chronic environmental exposure, if any, to EE2 on these species of amphibian.

6.3. Effects of exposure to 17α-ethynylestradiol

6.3.1. *Xenopus laevis*

To date there is little information available on the response of *X. laevis* to exposure to EE2. Preceding studies had shown that exposure to environmentally relevant concentrations of E2 (0.1-0.2 µg/L) during the period of sexual determination and differentiation could reverse the phenotypic sex of 50% of genetic male frogs (Lutz et al. 2008, Wolf et al. 2010). Since EE2 has a similar or greater potency than E2 (Folmar et al. 2002) and is more persistent (Ying et al. 2002), it was hypothesized that EE2 could affect *X. laevis* at similar or lesser concentrations. However, the current *X. laevis* study found that only 7 and 17% of genetic male *X. laevis* suffered from reversal of phenotypic sex when exposed to 0.84 and 8.81 µg EE2/L, respectively (Tompsett et al. 2012). As such, it was concluded that the effective concentration for feminization might be greater for EE2 than for E2, although the reasons for this difference are not completely clear. While there was a lesser degree of complete reversal of phenotypic sex than expected, a significant proportion of *X. laevis* underwent abnormal sexual development when exposed to EE2 and developed as mixed sex individuals with both testicular and ovarian tissues or developed as phenotypic males with abnormal testicular morphology. In the 0.09, 0.84, and 8.81 µg EE2/L treatments, these individuals accounted for 83% (11% mixed sex, 72% abnormal male), 87% (20% mixed sex, 67% abnormal male), and 75% (33% mixed sex, 42% abnormal male) of total genetic males, respectively. These proportions of mixed sex and abnormal individuals were greater than those observed after exposure to E2 (Hu et al. 2008, Lutz

et al. 2008, Wolf et al. 2010). Overall, a total of 83%, 93%, and 92% of genetic male animals in the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively, developed abnormally for their confirmed genetic sex as phenotypic females, mixed sex individuals, or abnormal males.

The transcriptional responses of genetic male *X. laevis* tadpoles to exposure to EE2 during sexual determination and differentiation were evaluated when tadpoles reached NF stage 53 by use of *Illumina* sequencing of the transcriptome coupled with an RNA-Seq expression analysis. Due to the significant workload, time investment and high costs of this type of analysis, these responses were only evaluated in tadpoles exposed to 0.84 µg EE2/L. The analysis showed that a number of biological processes were up- or down-regulated in exposed tadpoles as compared to solvent control tadpoles. The up-regulated processes included steroid and xenobiotic signaling and metabolism, biosynthesis of steroids, and gonadal development, while down-regulated processes included a different set of genes involved in steroid and xenobiotic signaling and metabolism, thyroid hormone signaling and metabolism, and testicular development.

Some of the significantly regulated processes are more meaningful when anchored to the phenotypic and biological data gathered in the group of individuals from the same exposure that were grown throughout the larval period and past metamorphosis in water containing EE2 (89 d). As described above, a total of 83%, 93%, and 92% of genetic male animals in the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively, developed abnormally for their confirmed genetic sex and were characterized phenotypically as either female, mixed sex, or abnormal male. Testicular development was affected in these individuals, which is in agreement with the down-regulation of transcripts involved in this process as observed in the RNA-Seq experiment. The individuals that were grown throughout development in EE2 also displayed significantly delayed metamorphosis as compared to animals from the control and solvent control treatments. The thyroid hormones are solely responsible for directing metamorphosis in the amphibians (Shi 2000), and the RNA-Seq analysis indicated that thyroid hormone signaling and metabolism, as well as the interconnected retinioic acid signaling pathway, were down-regulated at NF stage 53, which preceeds the period of metamorphic climax at NF stages 61-64. Thus, the biological effects of abnormal phenotypic development and delayed metamorphosis, which were not evaluated until after metamorphosis had been completed, were linked to molecular shifts at the transcriptome level that happened before the biological effects themselves were manifested.

The molecular effects of chronic (89 d) exposure to EE2 in *X. laevis* were also evaluated. Previous studies with *S. tropicalis* had indicated that chronic exposure to chemicals that interfere with normal sexual development past the period of sexual differentiation could lead to altered gene expression, and that the expression patterns were linked to the altered phenotypes induced by the chronic chemical exposure (Duarte-Guterman et al. 2010). In *X. laevis*, it was determined that some transcripts that are typically associated with exposure to estrogens, including *era* and *vtga2*, were found in greater abundances in individuals exposed to EE2. However, these alterations in abundances of transcripts were related to exposure to EE2 and not the phenotypic sex of the individuals exposed to EE2. It was hypothesized that the difference in effects between *X. laevis* and *S. tropicalis* were due to the relative sexual immaturity of *X. laevis* in the current study compared to the *S. tropicalis* in the previous study.

Greater abundances of *vtg* transcripts and VTG protein concentrations in liver and plasma, respectively, are well-characterized biomarkers of exposure to exogenous estrogens, including EE2 (reviewed by Sumpter and Johnson 2008). In the current study, abundances of transcripts of *vtga2* were greater in the livers of individuals that were exposed to all concentrations of EE2 evaluated. The transcripts of *vtg* present in the liver direct assembly of the VTG protein, which is a yolk-precursor. Normally, the VTG protein is then exported from the liver and deposited into the ovary, where it is incorporated into developing oocytes. However, male and juvenile individuals that have been exposed to EE2 also produce VTG (reviewed by Sumpter and Johnson 2008), and they do not necessarily have the endogenous processes or tissues to effectively sequester it. In the current study, VTG was localized by use of immunohistochemistry to the kidney-gonad complexes of individuals from all phenotype classes exposed to all concentrations of EE2 tested. The long-term biological consequences of this abnormal protein production and sequestration are not completely clear, but it was hypothesized that an inappropriate use of energy resources to produce VTG (Tompsett et al. 2012) coupled with the down-regulation of thyroid hormone-related processes previously described led to the significant delays to metamorphosis that were observed.

6.3.2. *Rana sylvatica*

While the effects of exposure of *R. sylvatica* to EE2 during sexual determination and differentiation had been characterized prior to the current study, the previous studies either lacked sufficient supporting data (Witschi 1951) or suffered from design problems like lack of replication (Mackenzie et al. 2003). The current study improved upon these past studies and was able to more fully characterize the phenotypic effects of exposure to EE2 on *R. sylvatica*. In addition, a novel transcriptional sequencing technique was utilized to gather and compile sequence information for *R. sylvatica*, which previously had little transcript data published. This data was used to evaluate transcriptional responses after chronic exposure to EE2 in the liver of *R. sylvatica* at metamorphic climax.

R. sylvatica was found to be moderately sensitive to male-to-female phenotypic sex reversal after exposure to EE2 in comparison with other species of amphibian. Using the data gathered from exposure of *R. sylvatica* to 1.08, 9.55 and 80.9 µg EE2/L, effective concentrations for feminization and partial-feminization of 50% of presumed genetic male frogs, or EC_{50} values, were determined. Within the limits of the dataset, the EC_{50} for complete feminization was determined to be 7.7 µg EE2/L (95% confidence interval 3.8-15.1 µg EE2/L) and the EC_{50} for partial feminization was determined to be 2.3 µg EE2/L (95% confidence interval 0.5-5.3 µg EE2/L). The addition of more data points along the concentration response curve would help to refine these values, especially the value for partial feminization. Since the least concentration of EE2 evaluated (1.08 µg EE2/L) caused the greatest proportion of *R. sylvatica* to develop with a mixed sex phenotype, it is possible that lesser concentrations of EE2 which were not evaluated could have feminizing effects on genetic male *R. sylvatica*.

The sequencing of a *R. sylvatica* RNA sample with an *Illumina* sequencer allowed assembly of a partial transcriptome for this species for the first time. Because of the nature of the sequencing experiment, the transcriptome that was assembled from this single sample had relatively short gene sequences and multiple sequences per gene that could not be assembled into larger gene sequences because they did not overlap. To be able to assemble a transcriptome with longer, independent gene contigs, more samples would need to be sequenced. However, even though the sequenced transcriptome is considered somewhat of an unfinished draft, the sequence information compiled was utilized to design primers for previously uncharacterized genes of

interest in *R. sylvatica*. Working primer sets were designed for a total of 8 genes that have been affected by exposure to estrogens or other endocrine active chemicals in other species of aquatic vertebrate. Of these 8 genes, transcripts of *vtga2, hdlbp*, and *dhcr7* were found to be significantly greater in *R. sylvatica* after chronic exposure to EE2.

Table 6.1: Comparisons of endpoints measured in juvenile frogs from *Xenopus laevis* and *Rana sylvatica* experiments

Endpoint	***X. laevis***	***R. sylvatica***
Concentrations of 17α-ethynylestradiol utilized	0.09, 0.84, and 8.81 µg/L	1.08, 9.55, and 80.9 µg/L
Duration of exposure	89 d	55-100 d
Stage of animals at sample collection	Post-metamorphic	Metamorphic climax
Time to metamorphic climax	Delayed	No effect
Growth (mass and length)	-	No effects
EC_{50} for feminization	-	7.7 µg/L
EC_{50} for partial feminization	~8.81 µg/L	~2.3 µg/L
Abnormal development of testes	Common at 0.09, 0.84, and 8.81 µg/L	Rare at 1.08 µg/L, absent at 9.55 and 80.9 µg/L
Transcript abundances altered by exposure	↑*era, ar, vtga2, fdps* ↓*fxra, fxrb*	↑*vtga2, hdlbp, dhcr7*
Transcripts abundances with similar responses to exposure	Up to ~100-fold greater abundances of *vtga2*	Up to ~280-fold greater abundances of *vtga2*
Presence of vitellogenin protein	Present in kidney-gonad complexes of exposed animals	Not present

6.3.3. Comparisons between *Xenopus laevis* and *Rana sylvatica*

The sexual differentiation and development of both *X. laevis* and *R. sylvatica* were affected by exposure to EE2 during the period of sexual determination and differentiation. However, the phenotypic responses exhibited by the two species differed, as did the relative sensitivity of each species to EE2 (Table 6.1). The development of the testes of *X. laevis* was adversely affected after exposure to concentrations of EE2 of 0.09, 0.84, and 8.81 µg/L. However, male-to-female phenotypic sex reversal was relatively rare at the concentrations tested (7% and 17% at 0.84 and 8.81µg EE2/L), and, due to the relatively small proportion of feminized *X. laevis* even at the greatest concentration tested, it was not possible to calculate an EC_{50} for either partial or complete feminization. In contrast, male-to-female phenotypic reversal of sex in *R. sylvatica* was observed in 7%, 61%, and 93% of presumed genetic males exposed to 1.08, 9.36, and 80.9 µg EE2/L, respectively, which correlates to an EC_{50} for complete feminization of 7.7 µg EE2/L. Since that concentration of EE2 is within the bounds of those utilized in the *X. laevis* experiment, it can be concluded *R. sylvatica* is more sensitive to complete feminization than *X. laevis*.

The effects of exposure to EE2 on partial feminization, or development of mixed sex phenotypes, in *X. laevis* compared to *R. sylvatica* is less clear. For *R. sylvatica*, the EC_{50} for partial feminization was 2.3 µg EE2/L, but the number of mixed sex individuals was the greatest at the least dose of EE2 tested. Thus, the actual EC_{50} value could be somewhat less than that calculated using the current data set. In contrast, the number of mixed sex individuals in the *X. laevis* experiment was greatest at the greatest concentration of EE2 tested, which indicates that even greater concentrations of EE2 might cause development of a greater proportion of individuals with mixed sex phenotypes. Altogether, exposure of *X. laevis* to 8.81 µg EE2/L at least partially feminized 50% of confirmed genetic males (33% mixed sex phenotypes, 17% female phenotypes). Thus, this concentration of EE2 could be considered an approximate EC_{50} value for partial feminization. In this case, *X. laevis* is less sensitive to partial feminization by EE2 than *R. sylvatica*.

While *X. laevis* is less sensitive than *R. sylvatica* to EE2-induced complete and partial feminization, it is more sensitive to abnormal testicular development after exposure to EE2. In *R. sylvatica*, the development of abnormal males was rare and was not dependent upon exposure

to EE2. The only two treatments with abnormal males in that experiment were the solvent control and 1.08 µg EE2/L treatments with 5% and 3% abnormal males, respectively. In contrast, the proportion of *X. laevis* that developed as abnormal males after exposure to EE2 was significantly greater than in control treatments. Specifically, 72%, 67%, and 42% of confirmed genetic males developed abnormally in the 0.09, 0.84, and 8.81 µg EE2/L treatments, respectively. Therefore, it can be concluded that *X. laevis* is more sensitive than *R. sylvatica* to alterations in testicular development when exposed to EE2.

The differences in sexual differentiation and development of genetic males after exposure to EE2 between *X. laevis* and *R. sylvatica* could be due to inherent differences between the two species. Even though both species are sensitive to feminization/demasculinization by potent estrogens, they have dissimilar systems of sexual determination. Both species exhibit genetic sex determination, but *X. laevis* has a ZW system of sex determination while *R. sylvatica* has an XY system of sex determination. While some effort has been extended to characterize the molecular signals that initiate and propagate sexual differentiation in *X. laevis* (Yoshimoto et al. 2008, Okada et al. 2009, Yoshimoto et al. 2010, Yoshimoto and Ito 2011), there is currently no information available about complimentary processes in *R. sylvatica*, or in any other species of frog with an XY system of sex determination. Thus, it is possible that differences in the processes of sexual determination and differentiation drive the differences in sensitivity to EE2 between *X. laevis* and *R. sylvatica*. In addition, previous studies have revealed that the sensitivity of different species of amphibian to potent estrogens can vary by several orders of magnitude, even when the system of sex determination is the same in those species (Pettersson and Berg 2007, Lutz et al. 2008, Wolf et al. 2010, Tompsett et al. 2012). This differential sensitivity might be due to species-specific factors that have yet to be characterized.

The abundances of transcripts of genes involved in steroid signaling and metabolism, cholesterol biosynthesis, and vitellogenesis were evaluated in the liver of both *X. laevis* and *R. sylvatica* after chronic exposure to EE2. Of the genes evaluated, it was found that *era, ar, vtga2,* and *fdps* transcripts had significantly greater abundances in *X. laevis* exposed to EE2, and *fxra* and *fxrb* had significantly lesser abundances in exposed *X. laevis*. Of these six genes, only *vtga2* transcripts showed a similar response in *R. sylvatica* exposed to EE2. The differences in significantly regulated transcripts between the two species could be due to a number of factors. First, the *X. laevis* individuals were evaluated after completion of metamorphosis (NF stage 66+)

while *R. sylvatica* were evaluated at the time of metamorphic climax, which coincides roughly with NF stages 61-64. In addition, *X. laevis* were all evaluated at the same time point after 89 d of exposure to EE2, while *R. sylvatica* were evaluated as individuals reached metamorphic climax, which ranged from to 55-100 d of exposure to EE2. Lastly, it is possible that the differential regulation of transcripts was due to species-specific differences in response to exposure to EE2.

Histological evaluation of the kidney-gonad complexes of *X. laevis* and *R. sylvatica* exposed to EE2 revealed that exposed *X. laevis* had VTG protein present in these tissues while exposed *R. sylvatica* did not. In *X. laevis*, VTG was found to be present in individuals belonging to all phenotype categories and exposed to all concentrations of EE2. However, evaluation of the kidney-gonad complexes of *R. sylvatica* revealed that none of the exposed frogs showed evidence of abnormal vitellogenin protein production. This difference could have been due to the relative immaturity of the *R. sylvatica* individuals, which were evaluated at metamorphic climax, compared to the *X. laevis*, which were evaluated after the completion of metamorphosis, or due to species-specific differences between *X. laevis* and *R. sylvatica*.

The last major difference between *X. laevis* and *R. sylvatica* observed after exposure to EE2 was the differential sensitivity of the two species to EE2-induced effects on metamorphosis. In the *X. laevis* experiment, the time required by individuals to complete metamorphosis was significantly greater after exposure to increasing concentrations of EE2 in a dose-dependent manner. However, there was no effect of EE2 on time to metamorphic climax in *R. sylvatica*. The timing of metamorphosis is dependent upon the balance of thyroid hormones in the larval frog (Shi 2000), and there was evidence in *X. laevis* that individuals at NF stage 53, which is before metamorphosis begins, already showed depressions in abundances of transcripts involved in thyroid hormone signaling and metabolism. While the status of the thyroid hormone axis was not monitored in the wood frog, it is possible that they did not show effects on metamorphic endpoints because they were not similarly affected by exposure to EE2.

6.3.4. Conclusions

Although both species displayed an overall trend of feminization/demasculinization, the responses of *X. laevis* and *R. sylvatica* to exposure to EE2 during development were shown to differ intrinsically. However, the sexual development and molecular responses of both species were shown to be affected by concentrations of EE2 that are within the realm of environmental relevance. In addition, metamorphic endpoints in *X. laevis* were shown to be affected by environmentally relevant concentrations of EE2. The habitat preferences of both of these species indicate that it is probable that they will be present in areas that are likely to receive estrogenic inputs from the surrounding environment. Thus, it is possible that wild populations of either of these species could be exposed to EE2, either alone or in combination with other estrogenic chemicals, in quantities great enough to cause alterations in sexual development and, in the case of *X. laevis*, metamorphosis. While the effects of altered sexual development or expression of estrogen-responsive genes on fertility and fecundity are not well-characterized, changes in these factors have the potential to affect individual and even population level fitness in negative ways. *X. laevis* and *R. sylvatica* are not currently among the species of amphibian facing declining population levels. However, they are subject to negative selection pressures like loss of habitat, biotic stressors, and abiotic stressors. In combination with chemical stressors such as environmental estrogens, these negative selection pressures have the potential to adversely affect the survival of species into the future.

6.3.5. Future work

While the experiments presented herein helped to refine and expand knowledge about the effects of larval exposure to EE2 in *X. laevis* and *R. sylvatica*, future experiments would be useful to more completely characterize the responses of these two species to chronic exposures to EE2. In particular, it would be useful to evaluate concentrations of EE2 not utilized by the current studies, especially lesser and greater concentrations (<0.09 and >8.81 µg EE2/L) for *X. laevis* and lesser concentrations (<1.08 µg EE2/L) for *R. sylvatica*. With the data gathered from such studies, it would be possible to calculate EC_{50} values for complete and partial feminization for *X. laevis* and to refine the values calculated here for *R. sylvatica*.

Before definite conclusions can be drawn about the possible effects of environmental exposure to EE2 and other potent estrogens in *X. laevis*, *R. sylvatica*, and other species of amphibians, it would be useful to more fully characterize actual environmental concentrations of the potent estrogens, including characterization of temporal variations. While this type of work would be time- and cost-intensive, it is probably the best way to characterize probable exposure. Even if large-scale studies are not feasible, it would be helpful to researchers to have more recent data available than what is currently reported in the literature. The best survey currently available (Kolpin et al. 2002a, Kolpin et al. 2002b) is over a decade old, and it is known that human and livestock population densities, which can vary by a great degree over 10 years, are major drivers of the presence of estrogenic compounds in the aquatic environment.

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